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PRINCIPAL INVESTIGATOR:
Brian L. Wickes, PhD

CONTRACTING ORGANIZATION:
University of Texas Health Science Center at San Antonio
San Antonio, TX 78229-3900

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BRIAN L. WICKES
wickes@uthscsa.edu

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14. ABSTRACT

The increasing frequency of fungal infections and broadening spectrum of organisms that cause these infections has placed a growing burden on clinical microbiologists. This burden is especially difficult for military clinicians and microbiologists due to locations and circumstances under which they must treat their patients, which often precludes access to expertise and/or technology. The goal of this proposal was to develop a universal sequence-based identification system for fungi. The aims of this study included 1) Creation of an internet-accessible sequence database for fungal identification, 2) Development of standardized protocols, including template preparation and sequencing, for these analyses, 3) Generation of reference sequences for the database, and 4) Confirmation of the utility of the database by comparison to current clinical protocols. All of these aims have been completed with the last aim representing a clinical study comparing our system to current microbiological methods for fungal identification. The results of this study, in which more than 500 clinical isolates were tested, showed that our method was significantly more accurate and faster than current microbiological methods. Importantly, the nature of our system, which entails a biocurated sequence database, allows this system to be updated regularly with novel sequences from organisms that we may not have in the database. We have also developed standard protocols, which include automated instrumentation that could be used in laboratory settings. The database is web accessible and utilizes publically available algorithms, but has been modified so that non molecular biologists as well as technicians without extensive mycological experience can access and use the system.

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Table of Contents

INTRODUCTION: The purpose of this proposal was to develop a system for the molecular identification of human fungal pathogens. This study addresses a major problem in clinical microbiology, which centers on the difficulty of identifying pathogenic fungi. Clinical microbiologists can easily identify common fungi; however, identification of rare fungi from clinical specimens cannot be reliably done without specific training in mycology. Unfortunately, individuals with this training are in short supply in both civilian and military hospitals. Importantly, the frequent nature of fungal infections in military personnel (trauma, burns, etc) can predispose patients to infections from both common and rarely seen fungi. The key aspect of our identification system consists of a biocurated database of DNA sequences derived from reference fungi, whose identity has been confirmed before adding the sequences to the database. Our system will enable laboratory technicians to make proper identifications without experience in mycology, using standardized techniques developed in this proposal, which can then be used to search an internet-accessible database developed by our laboratory. The output from searches of this database have reconfigured from the standard BLAST algorithm search output currently used by Genbank (the largest sequence repository in the world) to yield an accurate identification that utilizes proper and consistent fungal nomenclature, and also alerts users to any important information about the identity such as changes in nomenclature, or other information that could be clinically relevant. Our system will enable any clinical laboratory, regardless of mycological expertise, to identify any human fungal pathogen faster and more accurately than is presently possible, using a single assay.

BODY: This reporting period consists of the final report and summarizes all four tasks. At the completion of the funding period, all tasks have been successfully completed, with the final tasking including a clinical study comparing our methodology to current methodology. Each task will be briefly reviewed with the major findings discussed.

Task 1. CREATION OF AN INTERNET-ACCESSIBLE, SEQUENCE DATABASE FOR MOLECULAR IDENTIFICATION OF ALL KNOWN HUMAN FUNGAL PATHOGENS.

The main objective of this task was to develop a way to bring our database to the Internet, so that it would be accessible from anyplace that had computer access, including military hospitals or medical facilities. This objective had a number of components, which were divided into creation of the actual Internet portal (webpage), two databases of sequence and strain information (one located in our bioinformatics core to be used for the actual searches, and one on a desktop computer for data storage), and finally, embedding of the actual algorithms that allow sequence searching and manipulation.

The web portal for our database includes a domain at UTHSCSA with the website located at < http://pfris.uthscsa.edu/>. This site falls under UTHSCSA internet security. The site and its contents are only modifiable by us, are continually updated, and are backed up daily. The searchable component consists of the standard BLASTn window, where sequences from an unknown isolate are pasted. The web page provides all of the information about the system, which we have called PFRIS (Pathogenic Fungi rDNA Identification System), including how to

perform searches. Additional information via links to other sites and management of the site are included. **Figure 1** displays the web page that will serve as the portal to our database.

Fig. 1. PFRIS Webpage portal. The figure displays the web page that will be used as the portal into the PFRIS database. Information about the database is shown in the left hand column with links to take users to additional information. The description is shown in bold blue, and the button that takes users to the search function is listed as “Proceed to PFRIS”.

Embedded within the web page will be our searchable database. This database contains all the sequence and strain information for each isolate. Users can click on individual records to obtain this information when a specific record comes up in a search. These records are updated regularly as we obtain new information, or if we obtain a new strain that is not in the database, a new record can be easily created. In this way, the database is always kept current with the field of clinical mycology.

In addition to the actual database and web portal, we also have established and maintained a culture collection with multiple back ups, dispersed throughout the university. This collection contains frozen stocks of isolates that we have entered in the database. We continually add cultures to this collection as we obtain them as clinical isolates, or order them from culture

collections. Internal records separate from the PFRIS database also serve as a redundant back up for strain information. Depending on the original source of the strains, these strains can be available for study if they are not proprietary.

Task summary: In this task we have established a live fungal culture collection of isolates whose sequences have been obtained and deposited in our database. The database contains all information associated with each strain and is searchable through an integrated software package that uses the existing BLASTn sequence search algorithm. This information is found on the PFRIS website, along with other information about the database and ho to use it.

Task 2. DEVELOPMENT OF STANDARDIZED PROTOCOLS FOR PCR AND SEQUENCING TEMPLATE PREPARATION.

Throughout the course of this study we have attempted to develop standardized protocols for growing, preparing template DNA, and sequencing DNA from fungal isolates. We have established a standardized protocol for accomplishing this task. Because the major goal of this proposal was to develop a system that could be used by people with no formal training in mycology, a crucial step was to develop a simple, universal extraction procedure that could be applied to any fungus. A number of procedures were evaluated, and one procedure was settled on due to its ease of preparation and high PCR success rate. This procedure uses a commercial reagent from Applied Biosystems called Prepman Ultra, and simply requires a suspension of cellular material in the solution, heating for 15 minutes, and removal of a few microliters of supernatant, after centrifugation, that serves as PCR template DNA. After standardizing growth conditions and amount of material to prepare, a study was conducted to measure extraction success (denoted by generation of a PCR product). We conducted an initial study early on in the proposal and obtained a success rate of approximately 91%. We then made some modifications to the method and now have a method that works every time. Table 1 shows the results;

Table #1. DNA isolation success rate.

Morphology	No. Preps	Succeeded	Failed	% Successful
Yeast	155	155	0	100
Hyphal	391	391	0	100
Total	546	546	0	100

We have continued to develop this technique and have since moved onto automating the method using an instrument from Promega, Inc. The company has supported our efforts and we presented an abstract of our methodology earlier this year at the American Society of Clinical

Pathology Meeting (Abstract number ID60, "Automated DNA extraction RT-PCR based amplification for the detection of *Aspergillus fumigatus* in blood vs. serum specimens").

A second subaim consisted of developing a universal PCR reaction that would enable the amplification of sequences from any fungus. This step was important, obviously because each isolate would be unknown, and in many cases, the phylum that it belongs to could also be unknown, making genus-family-or even phylum specific PCR primer design not possible. Therefore, the only alternative was to insure that the PCR primers were indeed universal. The major aspect of this aim was to design universal primers and then develop a PCR mix, which would be able to reliably amplify template from any specimen, even pigmented isolates that contain PCR inhibitors. In order to design primers, we used the historic rDNA primers (2, 3) as a starting point and then downloaded the corresponding rDNA sequences, plus flanking regions, from the NCBI Entrez Genome Project site for fungal genomes. Contigs from approximately 50 fungal genomes were then searched with four primers most frequently used for amplifying fungal rDNA. The alignments revealed that the priming sites were 100% conserved, which confirmed that our choice of universal primers was correct. Importantly, this search included sequences from all four fungal phyla. Therefore, these four primers have been confirmed as being truly universal for amplification of fungal rDNA sequences.

Task 3. GENERATION OF TYPE SEQUENCES.

This task constitutes the bulk of the data gathering for this grant, and consists of obtaining sequences from fungal cultures. We expanded the acquisition of type sequences to include genome sequences, of which there are now hundreds, reference cultures, and clinical isolates that have already been identified. These isolates are collectively called references cultures, and the sequences obtained from them are called reference sequences. Reference sequences are defined as being obtained from cultures whose identity has been confirmed, and which provides the "biocurated" aspect of the database.

The sequences obtained from each culture have been placed on our desktop database, which serves as one back up, and from there, are uploaded onto the website database, which itself is also backed up. Prior to the upload, the sequence is rigorously examined including performing the appropriate molecular searches. In some cases, additional sequencing was done for confirmation. Since the attachment of the sequence to the appropriately named fungus is a key aspect of the database, we did extensive background work in determining the proper names to use, including consulting our over sight committee. If any aspect of this preparation was unclear or doubtful, the sequence was not uploaded. No problems with this strategy have been encountered and the process continues to this day, and will continue after the grant is complete. Importantly, continually adding sequences provides redundancy, which will help account for the natural strain-to-strain variation in a species. We have also added some new fields to each record to now include phylogenetic data obtained from the NIH, which broadens the amount of information that each record contains.

Data security has been incorporated into the database in a number of ways. Both the live cultures and the sequences obtained from them are backed up in redundant fashion. The live cultures are

stored frozen in three separate locations as sub cultures, and the sequences are stored in three separate locations, including back up drives. In summary, this task is largely a data-gathering task in which we obtained and banked as much data as possible. The number of sequences that we have is approaching two thousand, and will continue to grow indefinitely.

Task 4. CONFIRMATION OF THE UTILITY OF A MOLECULAR BASED IDENTIFICATION SYSTEM BY COMPARISON TO CURRENT IDENTIFICATION STRATEGIES.

This task constitutes the main goal of this study and directly addresses the main hypothesis that *that a sequence-based identification system will greatly improve fungal identification efficiency by replacing a variety of diagnostic tests, whose utility may be restricted by species, expense, patient status, or mycological expertise, with a single assay.* The task was conducted in conjunction with the Clinical Microbiology Laboratory at the University of Texas Health Science Center University Hospital. Briefly, the clinical laboratory would conduct their normal fungal identification using standard strategies that they normally use. The cultures would be saved until they obtained an identification and were finished with the isolates. We would then obtain the cultures and perform our identification using the PFRIS system. Time to identification was recorded for both methods, and identifications were compared once final identifications were made. Discrepancies were resolved by our collaborators in the UTHSCSA Fungus Testing Laboratory, which is certified for fungal identification by the College of American Pathologists. Data were then analyzed statistically for accuracy (both stringent to the species level, and genus only identifications, which were accepted as correct) turn around time.

A total of 509 isolates were tested by both groups (defined as University Hospital (UH), and our group (PFRIS)). Out of this total there were 81 mismatches (15.9% disagreement). The PFRIS method was incorrect for 5 isolates (0.9%), broken down as 2 isolates misidentified, 1 isolate with no matching sequencing in the database, and 2 isolates that had the correct identification, but below our standard cutoff values, so they were counted as incorrect. The UH method had 17 wrong identifications (3.3%) when the less stringent criterion was used. This criterion accepted the identification as correct if the identification was reported out as a genus only identification (i.e., Candida sp., vs. the more stringent *Candida albicans*). If the more stringent criterion was applied, the number of incorrect identifications by UH rose to 76 (14.9%).

We next compared the average time to identification. This comparison used the initial streaking of the primary plate as the T_0 time. The plate then is incubated and tests are conducted as soon as the culture produces enough cellular material. The UH method took, on average, 88 hours (mean = 72 hrs) to yield an identification, whereas our PFRIS method took 8.6 hours (mean 7.5 hrs) to yield an identification.

Finally we conducted a statistical analysis of these results using the paired t-test. Accuracy, using a genus only cutoff (the less stringent criterion) revealed that at the 95% confidence interval, the UH method was 95.53% and the PFRIS method was 99.02% accurate, with a significant difference ($P<.000017$), strongly suggesting that our method was significantly more accurate. At

the more stringent genus and species level, the PFRIS method was also much more accurate (99.0% vs. 81.1% for the UH method, $P<3.68 \times 10^{-21}$)

Our conclusions from the clinical study are that at a less stringent level where species identification is not always possible, our PFRIS method is significantly more accurate than the current clinical methodologies (UH), which are still very very good. However, at a more stringent level where species identification is required, the PFRIS method is still excellent while the current clinical methods are much less accurate (also significantly different). Finally, when a comparison of turn around time is made, we fell that this is where the PFRIS methodology has a massive advantage. The PFRIS method can identify a strain in slightly more than 8 hours, which includes any sequencing errors. If no sequencing errors occur, the identification can be done in a traditional work shift (7.5 hrs). The UH method was much much slower, taking on average almost 4 days. This difference, in our opinion, is the greatest impact of our study.

In the last part of the aim we have looked for ways to improve our system. To date we have added a number of additional programs that can act on the existing data in the database. These programs include a basic statistical analysis package, primer design program, sequence alignment, and phylogenetic analysis program. These programs were downloaded and then added to the PFRIS platform and will be available to users depending on their administrative status. The programs include the primer design tool from BLAST, the ClustalW program for multiple sequence alignments, and the PhyML3.0 program for phylogenetic analysis. We have also developed some of our own programs, which we use for "housekeeping functions". For example, by using standard SQL queries embedded in Hypertext Preprocessor (PHP), we can obtain basic statistical information about the current data in our PFRIS database (Ex: number of unique records, number of unique *Aspergillus fumigatus* records etc). This added functionality will be made available to users who are interested in the data in the database, and may have uses that include other platforms. Access can be controlled by our computational group, insuring the integrity of the data.

We continue to use this database for both diagnostic and research projects with goal being to have as large and as diverse a collection of clinically important fungi as possible with a focus on organisms that could be important to the US military. We have isolated fungi that could be potential zoonotic agents, and have used molecular sequencing to identify a number of these isolates from a wide range of animals (3, 5, 11, 14, 17). Given the presence of the United States military in Iraq and Afghanistan, we have also been interested in isolates that have origins in these regions and have published one report on a case involving an isolate from the middle east (18). Other studies have included the molecular identification of isolates that infect the immunosuppressed (2, 4, 7, 10, 12, 16), which are of interest because these are rare infections that can be impossible to identify and difficult to treat. We have even had success using the molecular approach to identify drug resistant bacteria (13). We also supported a genome-sequencing project centered at MIT that used one of our strains (9), and the data in the database have been useful for a number of basic research studies (1, 6, 8, 15).

KEY RESEARCH ACCOMPLISHMENTS: Most of the key research accomplishments have centered around publications, however, we have also established collaborations with military hospitals, made presentations, and continued to expand the programming aspect of this study, and validated our database. A brief list of key accomplishments is as follows:

A. We have continued to actively publish our results, which have put us in contact with a number of potential collaborators for grants. One military-based grant collaboration was funded. We consult on several other grants.

B. We have at least two publications that report clinical results for US servicemen.

C. This proposal supported a graduate student. She applied for a highly competitive American Society for Microbiology Clinical Fellowship, which is based at six universities in the US. She was offered five interviews. At the University of Pennsylvania Medical center, she was one of more than 1500 applicants. Six were interviewed, and she was selected as the top choice.

D. We have completed the final task of the study, which was the clinical comparison. Our method has proven to be more accurate, faster, and reduces the number of identification assays that need to be applied to a specific isolate. Consequently, we conclude that our method is a better method for fungal identification than all of the current methods utilized in a clinical laboratory.

REPORTABLE OUTCOMES:

I. The number of manuscripts generated by this study includes four in preparation, two submitted, one in press, twenty-two published. Two more are planned, which if accepted, will result in thirty journal publications. There was also one book chapter published.

II. Seventeen abstracts have been presented at local, regional, or national meetings.

III. Four invited seminars were presented:

Nov 2009 “Current issues in *Aspergillus* quantitative real time PCR (qRT-PCR) standardization.” Diagnostics of Invasive Aspergillosis: From Experimental Models to Clinical Evaluation. Combined IAAM and AsTeC Workshop, Bethesda, MD.

July 2009 “Human Fungal Pathogens: Keeping Nature’s Ultimate Consumers Away from the Table.”Voelcker Biomedical Research Program, San Antonio, TX.

January 2009 “The Changing Landscape of Fungal Identification: Morphology and Molecules”. UTHSCSA Dept. of Pathology Research Conference. San Antonio, TX.

April 2008 “What’s the Fungus!!? -- Can Molecular Identification Find a Place in the Clinical Mycology Laboratory?” San Antonio Center for Medical Mycology, San Antonio, TX

A fourth seminar by Anna Romanelli (graduate student supported by this project) was presented at the University of Texas, San Antonio, April 2010 entitled, “PFRIS: A biocurated database for fungal identification.”

IV. Additional Funding During Research Period.

“Detection and significance of antifungal resistance in oropharyngeal candidiasis”. PI, Tom Patterson, MD, Chief, Infectious Disease and Professor, Dept. of Medicine, University of Texas Health Science Center at San Antonio. Sponsor: NIH, National Institute of Dental and Craniofacial Research (NIDCR). Awarded 07/06. Role: Co-I.

“Laser Microdissection (LMD) with DNA PCR Amplification and Sequencing: A Novel Method for Determining the Etiology of Fungal Burn Wound Infection” PI, Davignon, Laurie, Major, MD, Maj, USAF, MC, Assistant Chief, Infectious Disease Service, San Antonio Military Medical Center, Fort Sam Houston, TX 78234-6200. Sponsor: Brooks Army Medical Center-Fort Sam Houston. Awarded 10/08. Role: Consultant.

“Animal Models of Infectious Diseases” PI, Tom Patterson, MD, Chief, Infectious Disease and Professor, Dept. of Medicine, University of Texas Health Science Center at San Antonio. Sponsor: NIH, National Institute of Allergy and Infectious Diseases. Awarded 03/2010 Role: Consultant.

“Small Animal Model Development and Utilization for Target Identification and Testing of Diagnostics, Therapeutics and Vaccines for Selected Invasive Fungal Diseases. PI, Tom Patterson, MD, Chief, Infectious Disease and Professor, Dept. of Medicine, University of Texas Health Science Center at San Antonio. Sponsor: NIH, National Institute of Allergy and Infectious Diseases. Awarded 09/2010 Role: Consultant.

V. Publications To date: (Four in preparation, Two submitted, One in press. Twenty-two published)

Calvano, T.P., Blatz, P.J., Vento, T.J., **Wickes, B.L.,** Sutton, D.A. and Duane R. Hospenthal. 2011. *Pythium aphanidermatum* infection following combat trauma. (In Preparation)

Devers, K., Chohan, M.O., Rehman, T.U., Thornton, K., Young, S., **Wickes, B.L**., Yonas H., and R.Medina-Flores. 2011. Likely travel-associated CNS toruloma in an immunocompetent host caused by *Cryptococcus gattii*. (In Preparation)

Rajeev, S., Ilha, M., Harrison, J., Clifton, G., Grooters, A., Fu, J., **Wickes, B.L**., and D.A. Sutton. 2011. *Lagenidium* sp. infection in a dog. (In Preparation)

Marancik, D.P., Berliner, A.L., Cavin, J.M., Clauss, T.M., Dove, A.D.M., Sutton, D.A., **Wickes, B.L**., and A.C. Camus. Disseminated fungal infection in two species of captive shark. 2011. (In Preparation)

Haley, S.L., Long,, S.W., Romanelli, A.M., **Wickes, B.L.,** and R. Olsen. Ocular infection due to *Aspergillus sydowii*, an emerging human pathogen infection. (Submitted).

Pineda, C., Kaushik, A., Kest, H., **Wickes, B**., and A. Zauk. Maternal Sepsis, Chorioamnionitis and Congenital *Candida kefyr* Infection in Premature Twins. (Submitted).

Balasingham, S., Chalkias, S. Balasingham, A., Saul, Z., Bia, F., **Wickes, B.L**., and D.A. Sutton. 2011. The first reported case of a bovine valve endocarditis with *Engyodontium album*. In Press. Med. Mycol.

Kimura, M., Yaguchi, T., Sutton, D.A., Fothergill, A.W., and **B. L. Wickes**. 2011. Disseminated human conidiobolomycosis due to *Conidiobolus lamprauges.* J. Clin. Mic. 49:752-6

Mershon-Shier, K.L., Deville, J.G., Delair, S., Fothergill, A.W., **Wickes, B.L**., De Hoog, G.S., Sutton, D.A., and M.A. Lewinski. 2010. Fluconazole-resistant *Aureobasidium pullulans* var. *melanigenum* fungemia in a pediatric patient. Med Mycol. 49:80-83.

Metry, C.A., Hoien-Dalen, P.S., Maddox, C.W., Thompson, E. H., Sutton, D. A., Romanelli, A.R., **Wickes, B.L**., and A.L. MacNeill. 2010. Subcutaneous *Mycoleptodiscus* Infection in an immunosuppressed dog. J. Clin. Mic. 48:3008-3011.

Pariseau, B., Nehls, S., Ogawa, G.S.H., Sutton, D.A., Romanelli, A.M., and **B.L. Wickes**. 2010. *Beauveria* keratitis and biopesticides: A morphological and molecular comparison. Cornea 29:152-8.

Romanelli, A.M., Sutton, D.A., Thompson, E.H., Rinaldi, M.G., and **B.L. Wickes**. 2010. Sequence-based identification of non-sporulating basidiomycetous fungi from clinical specimens, a cautionary note. J. Clin. Mic. 48:741-752.

Menon, T., Herrera, M., Shankar, P., Palanivel, V., Rajasekaran, S., and **Wickes BL**. 2010. Oral candidiasis caused by *Kodamaea ohmeri* in a HIV patient in Chennai, India. Mycoses 53:458-459.

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3. Automated DNA Extraction RT-PCR based Amplification for the Detection of *Aspergillus fumigatus* in Murine Blood vs. Serum Specimens. 2010. Herrera, M., **Wickes, B.L**., Najvar, L.K., Kirkpatrick, W.R., Wiederhold, N.P., and T. F. Patterson. Annual Association for Molecular Pathology Meeting. San Jose, CA.

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VII. Book Chapters.

1. Systematics of the Genus *Cryptococcus* and Its Type Species *C. neoformans.* 2010. Kwon-Chung, K.J., Boekhout, T., **Wickes, B.L**., and J.W. Fell. In *Cryptococcus: From Human Pathogen to Model Yeast*. 2010. J. Heitman Eds. ASM Press, Washington, DC

VIII. Salaried Research Team Throughout the Course of the Study

Brian Wickes, PhD
Jianmin Fu, PhD
Monica Herrera, MD
Jazmin Ruiz, BS
Julia Taylor, BS
Anna Romanelli, PhD
Bobby Cortez, BS
Zachary Ritchey, BS
Borries Demeler, PhD
Annette Fothergill, BS
Michael Rinaldi, PhD
Deanna Sutton, PhD
Jeremy Mann, BS

IX. Notable outcomes of trainees who have moved on to other positions.

Anna Romanelli earned a prestigious American Society for Microbiology Clinical Fellowship award, and is now a clinical fellow at the University of Pennsylvania Medical Center.

Jazmin Ruiz is now a high school biology teacher.

Bobby Cortez is in the UTHSCSA Dental School, and will receive his DDS degree in May 2011, and PhD in May 2012.

Zach Ritchey is now in graduate school.

Monica Herrera will be leaving for a medical residency in Pathology this summer.

CONCLUSION: The final year of this study applied the database for fungal identification of isolates that have come to our university for identification, and in a direct head to head comparison of hospital isolates. In all aspects, the database has performed well. It has proven to be faster and more accurate than current hospital microbiology laboratory methods. We will continue to expand the utility of the database by adding functionality through tethering additional programs to the database that allow routine and advanced interrogation of the database. We have continued to publish, present data at meetings, and most importantly, the graduate student who has performed much of the work in this study has entered an excellent post-graduate Fellowship.

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Catheter-Related Fungemia Due to *Candida thermophila*

Maskit Bar-Meir,[1] Deanna A. Sutton,[2] Brian Wickes,[2] Cletus P. Kurtzman,[3] Stewart Goldman,[1] and Xiaotian Zheng[1]*

Children's Memorial Hospital, Northwestern University Feinberg School of Medicine, Chicago, Illinois 60614[1]; University of Texas Health Sciences Center, San Antonio, Texas 78229[2]; and National Center for Agricultural Utilization Research, ARS, U.S. Department of Agriculture, Peoria, Illinois 61604[3]

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We report a case of bloodstream infection caused by *Candida thermophila*, a yeast not previously associated with human disease. The infection occurred in a 13-year-old boy with medulloblastoma who presented with 1 day of fever. Multiple blood cultures were positive for yeast. Removal of the catheter resulted in prompt resolution of the fever and sterilization of the blood cultures. The species was identified by sequencing domains 1 and 2 of the large subunit rRNA gene. Antifungal susceptibility testing was also performed.

CASE REPORT

A 13-year-old boy with medulloblastoma presented to the emergency department because of a 1-day history of fever up to 39.3°C, decreased oral intake, and increased fatigue. The tumor was diagnosed 14 months prior to his presentation. The patient was treated according to the Children's Oncology Group A-9961 protocol with surgical resection followed by reduced-dose craniospinal irradiation and alternate cycles of cisplatin, vincristine, and cyclophosphamide. The last cycle was given 3 weeks prior to his presentation. A central venous catheter, in place for a year, was used for administration of chemotherapy and hyperalimentation. The patient also received *Pneumocystis jiroveci* pneumonia prophylaxis with trimethoprim-sulfamethoxazole (160 mg of the trimethoprim component twice daily for three consecutive days each week).

Physical examination showed a febrile but otherwise well-appearing boy. The central line site showed no signs of infection or inflammation. Total white blood cell count was 4,300/mm^3 with 3,400 neutrophils/mm^3 and 560 band forms/mm^3, hemoglobin was 8.1 g/dl, and platelets were 59,000/mm^3. Findings on a chest radiograph were normal. A blood culture was drawn from the central line; the patient was given a dose of ceftriaxone and was sent home. The blood culture grew yeast after 24 h, and the patient was called and admitted to the hospital. At that time he was still well appearing and afebrile. An additional set of central and peripheral blood cultures was obtained, and administration of intravenous liposomal amphotericin (AmBisome) at 200 mg (5 mg/kg of body weight) once a day was begun. Altogether, eight sets of standard blood cultures (BACTEC Peds plus/F and standard anaerobic/F for each) and four sets of fungal blood cultures (ISOLATOR 1.5; Wampole Laboratories) were drawn over a 5-day period, and nine (five of the standard blood culture bottles and all fungal cultures) grew yeast. Sterilization of the blood was achieved only following removal of the central venous catheter on the fifth day of the antifungal therapy. The patient completed 6 weeks of liposomal amphotericin therapy and recovered without complications.

Laboratory findings. The yeast isolate from the patient grew after 24 to 48 h of incubation at 37°C. The colonies were moist and white in color. The germ tube test was negative. No hyphae or pseudohyphae were observed. The isolate was evaluated by the Microscan Walkaway system with a yeast identification plate (Dade Behring) and the API 20C AUX system (bioMerieux). Both gave an identification of *Hansenula polymorpha*. When biochemical reactions were run independently of the rapid systems, the isolate was negative for urease and positive for nitrate and glucose. The yeast grew at 37 and 42°C but not at 50°C.

Since the yeast could not be identified satisfactorily with the Microscan and API identification systems, DNA sequencing was conducted to provide identification. The isolate was identified as *Candida thermophila* (9) from its unique DNA sequence in domains 1 and 2 (D1/D2) of the large subunit rRNA gene by the National Center for Agricultural Utilization Research in Peoria and by the University of Texas Health Sciences Center in San Antonio. As described earlier (5, 6), genomic DNA was extracted from the yeast cells and combined with primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5′-GGTCCGTGTTTCAAGACGG) in a PCR. The resulting D1/D2 amplicon of ca. 600 nucleotides in length was purified, and both DNA strands were sequenced using primers NL-1 and NL-4 and an ABI (Applied Biosystems) automated DNA sequencer. The GenBank accession number for this sequence is DQ402185. The sequence of the isolate differed from that of *Candida thermophila* (GenBank accession AF283568) by one nucleotide. Other phylogenetically closely related organisms included *Pichia salicis* (GenBank accession AF403148; 99% identity), a presently undescribed species, and *Pichia angusta* (GenBank accession U75524; 98% identity) (4, 6). Our isolate has been deposited with the ARS Culture Collection as NRRL Y-27863 and with the American Type Culture Collection (ATCC MYA-3665).

Antifungal drug susceptibility testing was performed by the broth microdilution method based on the CLSI (formerly NCCLS) guidelines (8). Briefly, RPMI medium was used. The inoculation size was 5×10^4 CFU. MICs were read

* Corresponding author. Mailing address: Children's Memorial Hospital, Northwestern University Feinberg School of Medicine, 2300 Children's Plaza, Box 53, Chicago, IL 60614. Phone: (773) 880-6910. Fax: (773) 880-4687. E-mail: x-zheng@northwestern.edu.

TABLE 1. MIC and minimum fungicidal concentration results

Drug	MIC (μg/ml) at 24 h	MIC (μg/ml) at 48 h	MFC[a] (μg/ml)
Amphotericin	≤0.03	0.125	0.25
Caspofungin	0.12	0.5	1
Flucytosine	0.12	2	≥128
Fluconazole	1	2	16
Itraconazole	0.06	0.25	0.5
Ketoconazole	≤0.03	0.125	1
Voriconazole	≤0.03	≤0.03	≤0.03

[a] MFC, minimum fungicidal concentration.

at 24 and 48 h by comparing the turbidity of test wells to that of the untreated controls. A change in turbidity equal to or greater than 90% compared to drug-free control results was used to establish MIC breakpoints. The minimal fungicidal concentration results were obtained by recording colony counts on plates. Results are summarized in Table 1.

Discussion. Invasive candidiasis is an important cause of morbidity and mortality in chronically or critically ill patients (2, 3). Infections caused by *Candida* species are the fourth most common cause of nosocomial bloodstream infection in the United States (1, 11), with species other than *Candida albicans* emerging as pathogens. The non-*C. albicans* yeasts are often associated with resistance to antifungal azoles and with higher mortality. We describe the first reported case of *Candida thermophila* causing a human infection.

C. thermophila was described as a thermophilic soil yeast capable of growth at 50°C (9). Although the current isolate did not grow at 50°C, it did grow well at 37 and 42°C. Since it is difficult to identify this species with either commercial or conventional biochemical assays, this characteristic of growth in elevated temperature can be an indication for further analysis such as rRNA gene sequencing. Identification of yeasts from the large subunit rRNA gene D1/D2 sequence comparisons has been highly reliable. Strains of the same species ordinarily show only 0 to 3 nucleotide differences (6), but a few exceptions to this pattern have been found. For example, *Candida guilliermondii* and *Candida fermentati* differ by 3 nucleotides in large-subunit D1/D2 but show only 40% relatedness when compared by nuclear DNA reassociation (10). Consequently, these two taxa are closely related but not conspecific. The current isolate was identified as *C. thermophila* based on its close genetic similarity to the type strain of this species. The single nucleotide difference with the type strain has been interpreted as intraspecies strain variation. Gene sequence analysis has been successfully used for the identification of pathogenic fungi in addition to analysis of morphological and biochemical characteristics (7).

Although this is the first reported case of *C. thermophila* causing candidemia in a human, this may not be the first case of invasive disease due to this recently identified yeast, because identification and differentiation of yeasts on the basis of morphological and biochemical characteristics can be difficult. Therefore, the incidence and prevalence of this organism and its pathogenic role might be underestimated.

Many *Candida* species causing invasive infections have been non-*C. albicans* yeasts such as *C. krusei* and *C. glabrata*. These species can be inherently (primarily) or secondarily resistant to fluconazole and may be more difficult to treat. The isolate from our patient was susceptible to all antifungals in vitro, and the patient was treated successfully with liposomal amphotericin, although fluconazole might have been as effective.

In summary, as the population of immunocompromised hosts grows, organisms previously not considered as pathogens might cause invasive disease. *C. thermophila* should be added to the long list of yeasts that can cause bloodstream infections in the immunocompromised or critically ill patient.

Nucelotide sequence accession number. The sequence of the D1/D2 amplicon described in this study has been deposited under GenBank accession no. DQ402185.

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Cutaneous Infection Caused by *Macrophomina phaseolina* in a Child with Acute Myeloid Leukemia▽

Ashok Srinivasan,[1,3,6]* Brian L. Wickes,[4] Anna M. Romanelli,[4] Larisa Debelenko,[2] Jeffrey E. Rubnitz,[1,6] Deanna A. Sutton,[5] Elizabeth H. Thompson,[5] Annette W. Fothergill,[5] Michael G. Rinaldi,[5] Randall T. Hayden,[2] and Jerry L. Shenep[3,6]

Departments of Oncology,[1] Pathology,[2] and Infectious Diseases,[3] St. Jude Children's Research Hospital, Memphis, Tennessee; Department of Microbiology and Immunology[4] and Fungus Testing Laboratory, Department of Pathology,[5] University of Texas Health Science Center at San Antonio, San Antonio, Texas; and Department of Pediatrics, University of Tennessee Health Science Center, Memphis, Tennessee[6]

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We report a case of *Macrophomina phaseolina* skin infection in an immunocompromised child with acute myeloid leukemia, which was treated successfully with posaconazole without recurrence after a hematopoietic stem cell transplant. The fungus was identified by DNA sequencing using both the internal transcribed spacer and D1/D2 region of the 28S ribosomal DNA gene.

CASE REPORT

A 6-year-old Caucasian girl was diagnosed with acute myeloid leukemia carrying the t(11;19) translocation. She underwent a matched, unrelated hematopoietic stem cell transplant (HSCT) because of increasing minimal residual disease but suffered a relapse in her bone marrow 5 years later. She was then reinduced with a 5-day course of clofarabine, etoposide, and cyclophosphamide. Following this course of chemotherapy, she was discharged from the hospital and placed on prophylactic voriconazole (VRZ), vancomycin, ciprofloxacin, and trimethoprim-sulfamethoxazole. Three weeks later, she presented as an outpatient with tenderness and erythema of a few days' duration above the right medial malleolus.

She had no complaints of fever or leg pain and had sustained no trauma to the right leg. On examination. she was afebrile and looked well. Her temperature was 37.1°C, pulse was 101 beats/min, respiratory rate was 20/min, and blood pressure was 95/56 mm Hg. Her lungs were clear to auscultation, and examination of her abdomen revealed no tenderness or hepatosplenomegaly. She had a 2-by-2-cm erythematous, papular, nonulcerative, tender lesion above the right medial malleolus with no induration or inguinal lymphadenopathy. There were no other skin lesions. Her complete blood count revealed a white cell count of 0.1 cells/μl (range, 1,800 to 8,000 cells/μl), platelet count of 17,000/μl (range, 170 to 450,000 cells/μl), and hemoglobin of 9.1 g/dl (range, 12 to 16 g/dl). Blood cultures showed no growth. The lesion progressed over the next week on antibiotic and antifungal therapy with meropenem, vancomycin, and VRZ. It became indurated, with a central necrotic eschar and a surrounding rim of erythema, and extended into the subcutaneous tissue. She continued to appear well during this period. Magnetic resonance imaging of the right lower tibia and fibula revealed nonenhancing edema of the skin and subcutaneous fat involving the medial distal right lower leg without muscle or bone involvement or any drainable fluid collection. Computerized tomography of the chest and abdomen revealed no evidence suggestive of any fungal lesions.

A punch biopsy was performed through the central ulcerative lesion. The histology showed focal epidermolysis and dermal necrosis accompanied by predominantly histiocytic infiltrate and microhemorrhages. Histopathologic examination with periodic acid-Schiff and Gomori methenamine silver stains revealed abundant infrequently branching septate hyphae in the epidermis and dermis, invading the blood vessels (Fig. 1). There was no pigment seen on the hyphal walls on hematoxylin-eosin or melan A staining.

The biopsy specimen was sent for bacterial and fungal cultures, but the quantity of the tissue was so small that it could not be ground and plated on standard fungal media. Bacterial and fungal cultures were performed with the thioglycolate broth only. Filamentous growth at the top of the thioglycolate broth was detected on day six and was subcultured on potato dextrose agar. The potato dextrose agar grew a mold with a black center and a white periphery. The reverse of the mold was black. Microscopically, it showed septate dematiaceous hyphae with dark sac-like structures.

After 6 days of incubation at 24°C, the media displayed a black mold which was septate and dematiaceous but difficult to identify by morphology alone. The isolate was submitted to the Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center at San Antonio (UTHSCSA), for further identification and antifungal susceptibility testing and accessioned into their stock collection as UTHSC 08-1505. There, the isolate was subcultured on potato flakes agar prepared in-house) (14) for in vitro susceptibility testing, temperature studies (24, 35, and 40°C), and determination of macroscopic and microscopic features. It was also subcultured on homemade V-8 juice (8) and carnation leaf agars (13) in an attempt to induce development of diagnostic morphological features.

* Corresponding author. Mailing address: Department of Oncology, St. Jude Children's Research Hospital, 262 Danny Thomas Place, MS 260, Memphis, TN 38105. Phone: (901) 595-4720. Fax: (901) 521-9005. E-mail: ashok.srinivasan@stjude.org.

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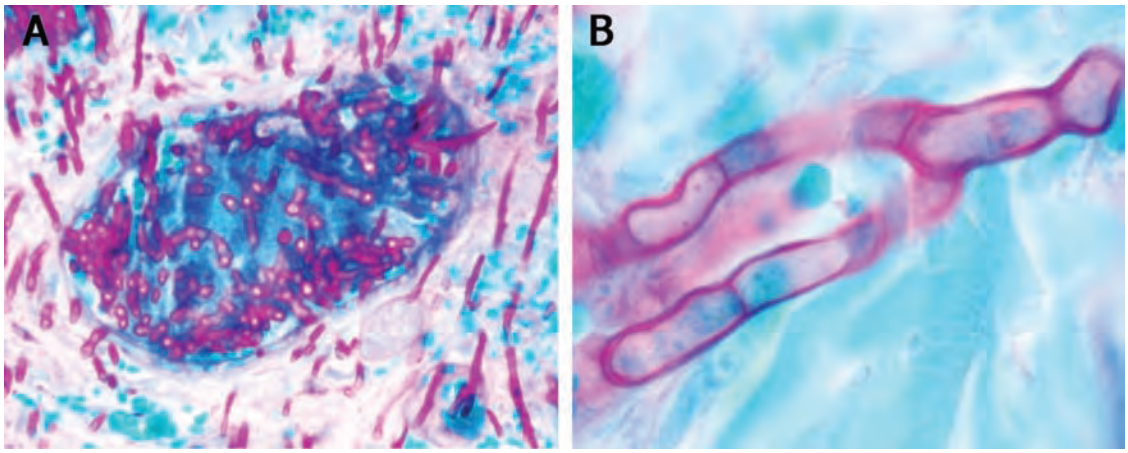

FIG. 1. Biopsy of skin lesion showing hyphae infiltrating a small dermal vein (located in the center of the image; periodic acid-Schiff stain; magnification, ×40) (A) or thick-walled septate hyphae showing infrequent branching (periodic acid-Schiff stain; magnification, ×100 under oil immersion) (B).

After 4 days' incubation at 24°C, all media displayed small brownish-black dots embedded in the media. Microscopically, they were initially similar to developing papulospores seen in the genus *Papulaspora*; however, as they matured and became more organized into structures, they were thought to possibly represent sclerotia, young conidiomata, or ascomata (Fig. 2). After 12 days, colonies on potato flakes agar were gray and effuse, with a dark diffusing pigment and the small black dots, which failed to develop into either conidiomata or ascomata and appeared to be sclerotia. Although the isolate failed to develop diagnostic structures, it did demonstrate growth at both 35°C and 40°C, confirming its pathogenic potential. Further incubation of media for a total of 3 weeks failed to produce fruiting bodies. The isolate has been deposited into the University of Alberta Microfungus Collection and Herbarium, a recognized culture collection, under the UAMH accession number 10953, for access by other investigators.

At this point, the culture was sent to the UTHSCSA Advanced Nucleic Acids Core Facility for molecular characterization. The isolate was grown for 24 h on potato dextrose agar at 30°C. Template DNA was prepared by lysing a small amount of cells in 50 μl of the Prepman Ultra reagent (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions. Five microliters of the pelleted Prepman super-

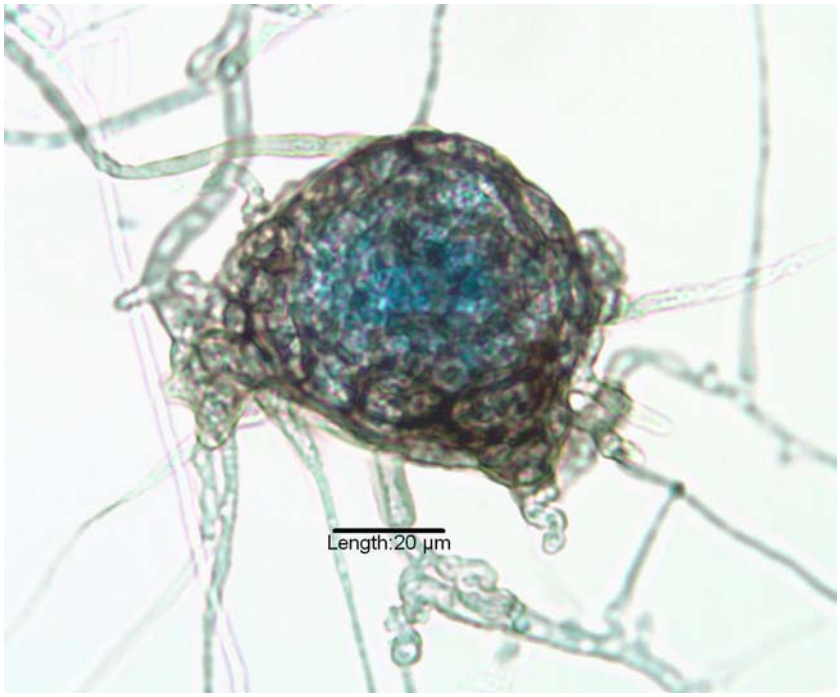

FIG. 2. Dark, immature sclerotium of *Macrophomina phaseolina* after 4 days of development on potato flakes agar at 25°C. Lactophenol cotton blue mount.

natant was used as a template for PCR. PCRs were prepared in a 50-μl reaction volume using high-fidelity *Taq* polymerase (Pfx 50; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The D1/D2 region of the 28S subunit was amplified with universal fungus primers NL-1 (5′-CATATCA ATAAGCGGA GGAAAAG-3) and NL4 (5′-GGTCCGTGT TTCAAGACGG-3) (10). The internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) was amplified using ITS1 (5′-T CCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGC TTATTGATATGC-3′) as described previously (16). PCRs were carried out in a PTC-100 thermocycler (MJ Research, Watertown, MA) using the preprogrammed three-step protocol as the standard program for all reactions, which consisted of 30 cycles using an annealing temperature of 58°C and a 1-min extension time. An aliquot of each PCR mixture was run on a 0.7% agarose gel to confirm amplification, followed by cleanup using a Qiaquick PCR purification kit (Qiagen, Inc., Valencia, CA). Fragments were sequenced in both directions at the UTHSCSA Advanced Nucleic Acids Core Facility. The sequence was then used to perform a nucleotide-nucleotide search using the BLASTn algorithm at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Hits were sorted on maximum identity, and identifications were made when BLAST searches yielded ≥98% identity. Both sequences returned BLAST hits with 100% identity for *Macrophomina phaseolina*. Results for the top three hits were *Macrophomina phaseolina* isolate IFAPA-CH724, 558/558 (100% identity), *Macrophomina phaseolina* strain MUCC532, 520/520 (100% identity), and *Macrophomina phaseolina* strain MUCC531, 520/520 (100% identity). The D1/D2 results for the top three hits were *Macrophomina phaseolina* strain CPC 11079, 614/614 (100% identity), *Macrophomina phaseolina* strain CPC 11070, 614/614 (100% identity), and *Macrophomina phaseolina* strain CPC 11052, 614/614 (100% identity).

Antifungal susceptibility testing was performed according to the Clinical and Laboratory Standards Institute M38-A2 microdilution format (4). Briefly, testing parameters included a final inoculum of 0.4×10^4 to 5×10^4 CFU/ml, consisting of hyphal fragments standardized spectrophotometrically, incubation at 35°C, and the use of RPMI-1640 medium (Hardy Diagnostics, Santa Maria, CA) for all agents except amphotericin B (AMB) (Bristol-Myers Squibb, New York, NY), which was tested in antibiotic medium 3 (Difco, Sparks, MD). Drug concentrations ranged from 0.03 to 16 μg/ml for AMB, posaconazole (PZC) (Pfizer, Inc., New York, NY), and VRZ (Pfizer), 0.015 to 8 μg/ml for caspofungin (CAS) (Merck, Rahway, NJ), and 0.004 to 2 μg/ml for terbinafine (TER) (Novartis, East Hanover, NJ). MICs were defined as the lowest concentration resulting in complete inhibition for AMB, PZC, and VRZ and 50% inhibition for TER. The minimum effective concentration (MEC), defined as the point where a visible change in the growth characteristic was noted, was read for CAS. The minimum lethal concentration was also recorded for AMB. *Paecilomyces variotii* MYA-3630 was included as a control organism. In vitro MICs/MECs (in μg/ml) at 48 and 72 h were as follows: AMB, 0.25/0.25; CAS, 1/1; PZC, 1/2; VCZ, 0.5/0.5; and TER, 0.06/0.25. The AMB minimum lethal concentration was 0.5 μg/ml. Although no breakpoints exist for this organism, all drugs demonstrated good in vitro activity as evidenced by MICs/MECs that were within achievable serum concentrations using standard dosing regimens (5).

Because the lesion had progressed during prophylaxis with VRZ, therapy was empirically changed to PZC prior to the availability of susceptibility test results. The lesion improved dramatically, with healing of the central lesion and resolution of the erythema, induration, and tenderness within 1 week, later accompanied by a recovery of her neutrophil count. Complete healing with central scarring was documented in 3 weeks. PZC trough levels were noted to be 1,190 ng/ml. Two months later, she underwent a matched unrelated transplant and received reduced-intensity conditioning with fludarabine, melphalan, thiotepa, and antithymocytic globulin. Micafungin was administered as antifungal prophylaxis during the conditioning and prior to engraftment on day +19, owing to the interaction of azoles with other medications and the presence of mucositis; PZC was restarted thereafter. The scarred lesion above her right ankle remained quiescent during the transplantation. She had no significant bacterial or fungal infections during the period of neutropenia and postengraftment while on prophylaxis with PZC. She continues to remain well 8 months after the transplantation, without evidence of graft-versus-host disease or leukemia.

Macrophomina phaseolina is an anamorphic (asexual) phaeoid mold in the ascomycetous family *Botryosphaeriaceae.* The binomial name *Macrophomina phaseoli* (Maubl.) Ashby was proposed by Ashby in 1927 (1) for the earliest pycnidial form on beans (*Phaseolus vulgaris*), from which the species epithet is derived (9). Another genus into which the organism had been placed was *Tiarosporella* (von Arx 1981) as *T. phaseoli* (6). Based on the most recent DNA sequence analysis of 113 isolates thought to align with the *Botryosphaeriaceae*, Crous et al. (6) divided the family into 11 clades and placed *M. phaseolina* as the only species in clade 3. This organism is also the coelomycetous synanamorph (another asexual form of the same fungus) of *Rhizoctonia bataticola.* Hence, our isolate also shows >99% identity with *R. bataticola* when a BLASTn search is performed (1). The species currently has no known teleomorph (sexual stage) and commonly produces only dark sclerotia in routine cultures. Crous et al., however, were able to obtain pycnidial formation and conidiation in their isolates on sterilized pine needles, thereby demonstrating the coelomycetous nature of this organism. Conidia lacked appendages and became brown at maturity. The presence of only sclerotia and the lack of any conidial formation in our isolate in culture are typical and made identification by morphological and/or physiological features alone impossible. This case is an excellent example to highlight the utility of combining molecular sequence data in otherwise sterile isolates with the cultural characteristics to obtain an unequivocal identification.

Macrophomina phaseolina causes charcoal root rot of soybean, cotton, chickpea, sorghum, corn, and other economically important crop plants (17). It has a wide host range, infecting about 500 plant species from more than 100 families around the world (11). The organism is soilborne, suggesting that our patient may have acquired the infection from an environmental exposure.

This is the first reported case of successful treatment of this infection in a patient, without recurrence, after HSCT. The only other reported case of human infection with *Macrophomina phaseolina* was found in an adult male following a renal transplant (15). The fungus was isolated from a purulent discharge from the left great toe and identified by molecular analysis. The target was the small subunit and ITS region of the rRNA gene. It was susceptible to several antifungal agents, and the patient's infection responded to treatment with VRZ alone. Subsequently he succumbed to invasive central nervous system infection with *Scytalidium dimidiatum*, a related species within the ascomycete family *Botryosphaeriaceae*, recently reclassified as *Neoscytalidium* (15).

Our patient had no signs of systemic fungal involvement and responded well to PZC therapy, to which it was susceptible in vitro. There was no evidence of recurrent infection despite administration of immunosuppressive medications. The good outcome in our patient may have been related to early diagnosis and treatment, an absence of dissemination, prompt recovery of the neutrophil count, and the absence of graft-versus-host disease posttransplant.

Rare and emerging opportunistic fungal pathogens are causing increasing morbidity and mortality in children and adults undergoing immunosuppressive therapy, including HSCT. These include species of *Candida* and *Aspergillus* other than *Candida albicans* and *Aspergillus fumigatus*; opportunistic yeast-like fungi, such as *Trichosporon* spp.; the zygomycetes; hyaline molds, such as *Fusarium* and *Scedosporium*; and a wide variety of dematiaceous fungi. Cutaneous fungal infections in patients with cancer may be either primary or a manifestation of fungemia. The main risk factor is prolonged and severe neutropenia. *Aspergillus*, *Rhizopus*, *Mucor*, and *Fusarium* spp. are the most common molds.

Among the dematiaceous molds, cutaneous infection with *Bipolaris spicifera* has been reported in a child with acute lymphoblastic leukemia (3), cutaneous alternariosis in an adult with acute myeloid leukemia (7), and infection with *Exserohilum rostratum* in a child with acute lymphoblastic leukemia (12).

This case emphasizes the importance of emerging fungal pathogens in immunosuppressed patients, including patients receiving prophylactic antifungal therapy. Although voriconazole demonstrated good in vitro activity, it failed to halt the progression of the lesion in vivo. Many of these emerging fungal pathogens are resistant to commonly used antifungal therapies.

The PCR-based DNA sequencing technique targeting the ITS region has been found to be a rapid and reliable tool to identify emerging pathogens of nonsporulating molds (2). This case highlights the importance of molecular methods in identifying these fungal pathogens.

Nucleotide sequence accession numbers. Sequences were deposited in GenBank under accession numbers FJ415067 for the ITS sequence and FJ415068 for the D1/D2 sequence.

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Case Report

Disseminated *Oxyporus corticola*infection in a German shepherd dog

C. W. BROCKUS*, R.K . MYERS†, J.M . CRANDELL‡, D. A. SUTTON§, B.L .WICKES+& K.K . NAKASONE#

**Charles River Laboratories, Preclinical Services, Reno, Nevada, †The Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames, Iowa, ‡Akron Veterinary Referral and Emergency Center, Akron, Ohio, §Fungus Testing Laboratory, Department of Pathology, and the +Department of Microbiology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, and the #Center for Forest Mycology Research, Northern Research Station, USDA Forest Service, Madison, Wisconsin, USA*

The filamentous basidiomycetous fungus, *Oxyporus corticola,* has not previously been reported in the human or veterinary medical literature. Identification of this organism as the etiologic agent of fungal osteomyelitis and multiorgan dissemination in a German shepherd dog was confirmed by comparison of ITS and D1/D2 sequences with known isolates.

Keywords *Oxysporus corticola*, canine, fungal osteomyelitis, disseminated mycosis

Introduction

Systemic fungal infections in dogs are uncommon and are typically caused by ubiquitous, opportunistic organisms. These infections can and frequently do disseminate, especially in young- to middle-aged female German shepherd dogs (GSD) [1]. The predisposition of GSD to systemic fungal infections, particularly aspergillosis caused by *Aspergillus terreus* [2] and *A. deflectus* [3,4], is thought to be associated with a hereditary immune defect [2,5,6]. This case involves a middle-aged female GSD infected with a fungus which has not been previously reported in the clinical literature, *Oxyporus corticola*. In addition, this case suggests that other, more unusual fungi, can also infect dogs, and that these fungi might be mistaken for *Aspergillus* species. Fungal identification and *in vitro* susceptibility testing are recommended for appropriate therapeutic management of disseminated mycoses.

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Correspondence: Charles W. Brockus, Charles River Laboratories, Inc, Preclinical Services, 6995 Longley Lane, Reno, NV 89511, USA. Tel: +1 775 682 2238; fax: +1 775 682 2100; E-mail: charles.brockus@crl.com

Case report

A 6-year-old female spayed German shepherd was referred to the Iowa State University (ISU) Veterinary Teaching Hospital for a painful boney mass on the right distal tibia that had caused the animal to limp for 4 weeks. On physical examination, right hind limb lameness was present with a palpable painful right tibia mass. Mild generalized lymphadenopathy also was noted. Medications and supplements that had recently been used included carprofen, (Rimadyl, Pfizer, Inc., Exton, PA), glucosamine, chondroitin, brewer's yeast, cod liver oil, and multivitamins. Laboratory data including a complete blood count (CBC), biochemistry profile, and urinalysis were unremarkable except for a mild hypercalcemia at 11.4 mg/dl (reference values 9.2–11.2 mg/dl). Serum was submitted for IgA concentrations and results were within reference intervals (190 mg/dl; reference intervals, 20–150). A proliferative mass was observed on the distal right tibia on radiographs (Fig. 1) but thoracic radiographs were within healthy parameters. Chorioretinal lesions of unknown cause were observed on retinal examination. A fine needle aspirate (FNA) and a biopsy specimen of the right tibial lesion were submitted for study. Macrophages with phagocytosed branching, septate hyphae with nearly parallel sides were observed in the FNA (Fig. 2) and fungal

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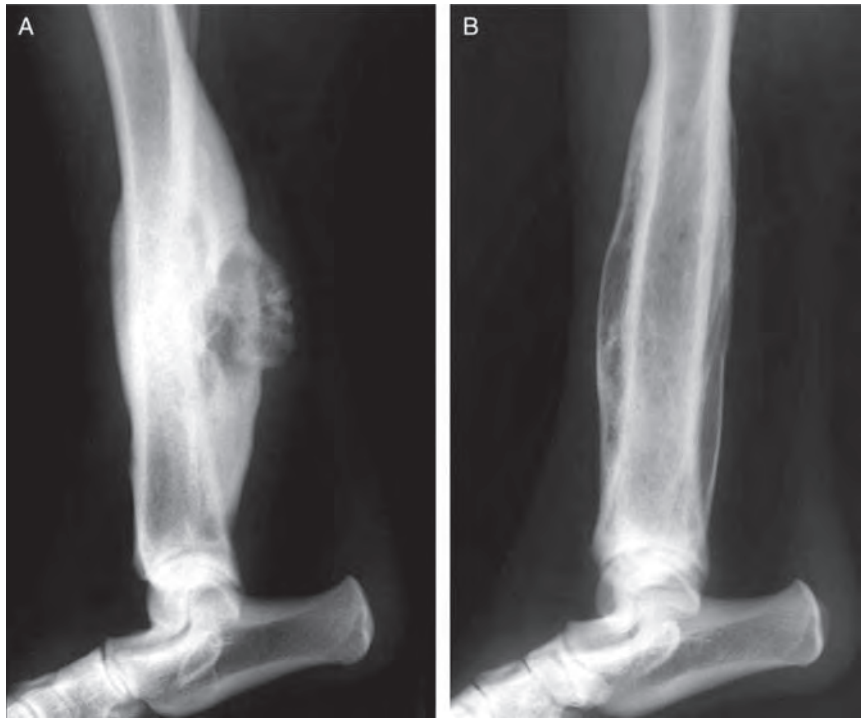

Fig. 1 An aggressive mixed productive and proliferative lesion is present in the mid/distal-tibia region (A). A smoother appearance but proliferation remained after 6 months of treatment. Productive changes have progressed distally (B).

hyphae were also noted in the biopsy specimen. Initially, this was interpreted as probable aspergillosis. Infrequent fungal hyphae were also present on a prescapular lymph node FNA indicating a disseminated infection. The biopsy specimen was submitted to the Iowa State University microbiology laboratory where portions were inoculated onto trypticase soy agar with 5% sheep blood (Remel, Lenexa, KS) and incubated aerobically and anaerobically at 35°C to detect bacterial pathogens. In addition, portions were cultured on Sabouraud dextrose agar (SDA; Difco, Inc., Detroit, MI) and incubated at 25 and 35°C for recovery of fungi. Rapid growth of a white, filamentous, unidentified fungus occurred in the aerobic bacterial cultures at 35°C and on the fungal cultures at both 25 and 35°C, but no bacterial growth was detected. The isolate from the SDA culture was then submitted to the Fungus Testing Laboratory at the University of Texas Health Science Center in San Antonio for fungal identification and *in vitro* antifungal susceptibility testing. The isolate was initially identified as a basidiomycetous fungus, and subsequently confirmed as *Oxyporus corticola* by sequencing.

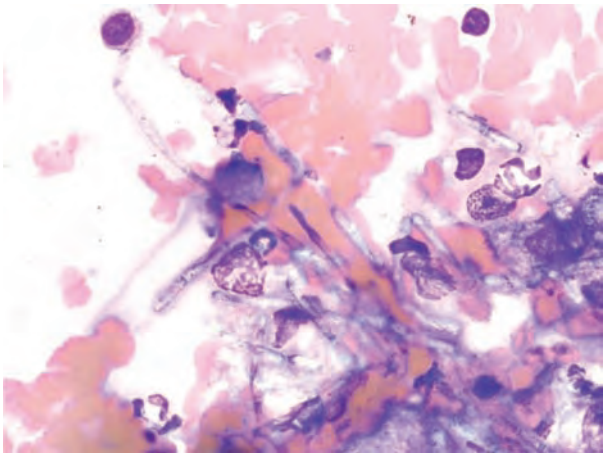

Fig. 2 Cytological photomicrograph of a cytocentrifuge fine needle aspirate specimen of the tibial lesion from the dog. Branching, septate, fungal hyphae with parallel walls were initially thought to be compatible with an *Aspergillus* species (Wright's stain).

The dog was administered oral compounded itraconazole (Sporanox, Janssen Pharmaceutica Products, Titusville, NJ) 200 mg BID. Two months after starting treatment, the patient's activity had improved, tibial pain was not present and limping was minimal, but radiographically the boney lesions had not changed. The CBC, chemistry profile, and urinalysis results were nearly identical to initial results except for a mildly increased total protein (7.4 g/dl: reference values 5.2–7.1 g/dl). Calcium was within reference values. Five months after initial presentation this dog and another in the same household presented with tetra-paresis identified as generalized lower motor disease. A toxin was suspected but never identified. Cerebrospinal fluid was taken from the fungal-infected dog

and was found to contain 90% non-degenerate neutrophils admixed with occasional macrophages and small lymphocytes, to have a mildly increased protein 38.3 mg/dl (ref. values 10–25 mg/dl) and a mildly increased creatine kinase (CK) at 14 mg/dl (ref. values 0.0–5.0 mg/dl). The total nucleated cell count was within reference values but the neutrophil percentage was markedly increased. There were also slightly elevated serum protein and CK concentrations indicating non-specific inflammation. Rapid improvement occurred and the dog was released on continued oral itraconazole therapy. A six month recheck revealed minimal change in right tibial mass and itraconazole was replaced with oral terbinafine hydrochloride (Lamisil, Novartis Pharmaceutical Corp., East Hanover, NJ) at 250 mg BID. At this visit the alanine transaminase (ALT) also had increased to 1,709 IU/l (reference values 24–105 IU/L) which was potentially attributed to recently-administered non-steroidal anti-inflammatory drug (NSAID), carprofen. As a result carprofen was then administered only as needed and the ALT gradually returned to reference values within 3 weeks. Ten months later the right distal tibia lesion had progressed down the limb into the joint with purulent inflammation observed in a hock joint cytological specimen (85% non-degenerate neutrophils) without observable microorganisms and negative microbiological studies. Amputation of the affected limb to remove the potential nidus of infection was suggested with a change of therapy to the use of amphotericin B but was refused. Terbinafine was discontinued and compounded itraconazole was re-administered orally at 200 mg BID. Periodic evaluations over the following 8 months revealed gradual worsening of boney lesions and progressively worsening ataxia. Computed tomography was performed and lesions were present in the left lower brain region, the lateral and third ventricles were enlarged, and the central vessels within the midbrain were deviated away from midline, all suggesting potential dissemination of the fungus to the central nervous system. One year and 8 months after presentation the dog was euthanatized due to progressive worsening of CNS signs. A necropsy was performed, but specimens were not collected for culture analysis in an effort to minimize the risk to laboratory personnel by an organism of uncertain pathogenicity to humans.

At necropsy, the mitral valve of the heart had rounded, nodular, to roughened margins. The endocardium of the pulmonary outflow track was roughened, granular, and pale in a small patch that proved to be granulomatous endocarditis and myocarditis. Liver was slightly enlarged and pale (steroid hepatopathy). Spleen had siderocalcific plaques. Bone changes within left and right tibias consisted of irregular thickening of the periosteum and cortices with multiple irregular pale nodules within the marrow (granulomatous osteomyelitis). In the brain, the left to mid thalamic area had a 2 cm dark tan circular lesion that pressed dorsally into the left ventricle (malacia).

Fungal hyphae were present in multiple organs and were demonstrated on the examination of routine hemotoxylin and eosin (H & E) histopathology stained sections, as well as on the special fungal periodic acid-Schiff (PAS) and Gomori's methenamine silver (GMS) stained material. Hyphae were generally fairly uniform in size bearing more or less parallel walls and frequent septa and occasionally perpendicular or dichotomous branching. In some areas the hyphae were slightly bulbous with undulating walls between the septa. In the heart, the endocardium, myocardium, and root of the aortic and mitral valves were thickened by the accumulation of large numbers of fungal hyphae, variable amounts of fibrosis, hemorrhage, hemosiderin containing macrophages, and relatively few macrophages. Hyphae were present without inflammation in the adventitia of the aorta. The periosteal arteries in both tibias were expanded by the accumulation of fungal hyphae, macrophages, neutrophils, fibrosis, and necrotic material throughout the wall. Some of these vessels were thrombosed. The medulla of the bone contained multifocal small granulomas containing fungal hyphae surrounded by macrophages and multiple small to very large areas of necrosis of bone surrounded by eosinophilic matrix containing fungal hyphae. One adrenal had a large nodule occupying most of the medulla and compressing the cortex which was composed of a necrotic center with either pale eosinophilic material containing fungal hyphae (Fig. 3) or large numbers of necrotic cells. The opposite adrenal had similar nodules obliterating most of the medulla and cortex. Both thyroid glands had multiple granulomas containing hyphae and multinucleate giant cells. The kidney had a few small random granulomas composed of macrophages and giant cells around fungal hyphae. Similar inflammation was present in the wall of renal and arcuate arteries along with fibrinoid necrosis of the media in one place and presence of fungal hyphae in the adventitia. The brain had an area of malacia in the mid brain corresponding to the gross description that was vaguely demarcated. The affected area was composed of vacuolated neurophils and swollen axons with multifocal areas of hemorrhage present around smaller vessels. Very few inflammatory cells were present and no fungal elements were detected.

Morphologic identification

The isolate submitted for identification was accessioned into the Fungus Testing Laboratory at the University of Texas Health Science Center at San Antonio as UTHSC 03-768. On potato flakes agar (PFA, prepared in-house) colonies were white, velvety to woolly, and displayed poor growth at 25°C in ambient air [7]. Tease mounts

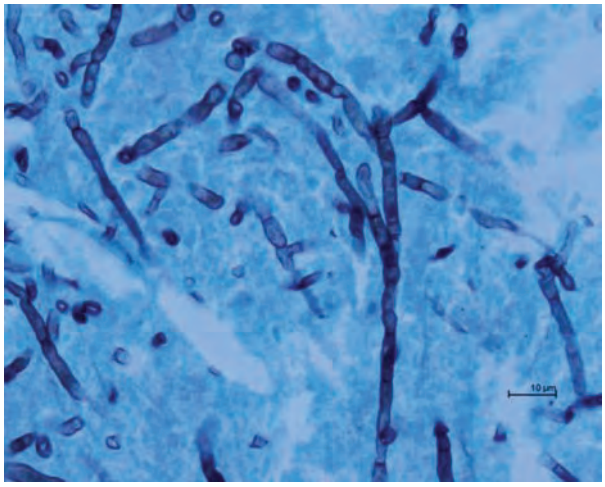

Fig. 3 Photomicrograph of the adrenal gland illustrating fungal hyphae disseminated throughout the gland (Gomori methenamine silver staining).

for microscopic features indicated only sterile, somewhat narrow hyphae. A slide culture on PFA at 25°C failed to grow and was repeated at 35°C. Colonies at 35°C remained white and sterile. The isolate failed to grow on media containing cycloheximide (Mycobiotic, Remel, Lenexa, KS), but did grow on benomyl agar prepared in-house [8]. Lack of growth on cycloheximide and growth on benomyl agar may be an indication that the fungus is a basidiomycete [8,9]. The isolate remained sterile, however, and hyphae lacked clamp connections sometimes seen with basidiomycetes. Furthermore, it failed to demonstrate spicules frequently produced by the basidiomycete *Schizophyllum commune* [9]. A lack of any diagnostic morphologic features prompted a molecular characterization under the accession number R-3610. The isolate has been deposited into the University of Alberta Microfungus Collection and Herbarium under the accession number UAMH 10739.

Molecular identification

DNA was prepared from sub cultures of R-3610, and the control isolates R-3716 (*Oxyporus populinus*), R-3714 (*O. corticola*), and R-3713 (*O. corticola*) obtained from the collection of one of us, K. K. N., at the Forest Products Laboratory, USDA Forest Service, Madison, Wisconsin, under culture collection numbers L-11146-sp, RLG-6775-sp, and RLG-4894-sp, respectively. The isolates were grown for 48–72 h at 30°C on potato dextrose agar (PDA; Difco, Detroit, MI). A small amount of hyphae and conidia were scraped off of each plate and suspended in 50 µl of Prepman Ultra reagent (Applied Biosystems, Foster City, CA). The suspension was heated for 15 minutes at 100°C and then pelleted for 5 min at maximum speed in a microfuge. The supernatant was transferred to a new tube and stored on ice until PCR reactions could be set up. PCR reactions were performed on 5 µl of the Prepman supernatant in a 50 µl PCR reaction using *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. ITS amplification products were obtained using the primers ITS1 and ITS4, and PCR conditions as previously described [10]. D1/D2 PCR products were obtained using primers NL1 and NL4, and PCR conditions as presented earlier [11,12]. Amplifications were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA) and amplicons were visualized by running a 15 µl aliquot of each PCR reaction on a 0.7% agarose gel and staining with ethidium bromide. The residual PCR product was prepared for sequencing by passing through a QIAquick PCR purification column (Qiagen, Valencia, CA). Purified templates were sequenced at the UTHSCSA Advanced Nucleic Acids Core facility using the same primers for ITS and D1/D2 amplification. Sequences were then used to perform individual BLASTn searches using the NCBI BLAST database and analyzed using MacVector software (Accelrys, San Diego, CA). Genbank accession numbers were assigned as follows: R-3610 ITS, D1/D2 (ACCESSION# EF011121, ACCESSION# EF011117), R-3713 ITS, D1/D2 (ACCESSION# EF011123, ACCESSION# EF011118), R-3714 ITS, D1/D2 (ACCESSION# EF011122, ACCESSION# EF011120), R-3716 ITS, D1/D2 (ACCESSION# EF011121, ACCESSION# EF011119).

A BLAST search of the R-3610 ITS and D1/D2 sequences was inconclusive. The ITS sequence showed closest similarity to *Oryza barthii*, but at an insignificant level (94% identity). The D1/D2 sequence showed the closest identity to an *Oxyporus* spp. (94%) and an *Oxyporus populinus* entry (also 94%); however, neither of these identities were significant matches. Three strains of *Oxyporus* species (*O. populinus*, R-3716 and *O. corticola*, R-3713, R-3714) from our culture collection were then compared to R-3610 by ITS and D1/D2 sequence. R-3713 and R-3714 (*O. corticola*) showed 100% identity to each other for both sequences (648/648 for the D1/D2 sequences, 639/639 for the ITS sequence). R-3610 was 99.85% identical to both of these isolates (647/648) for the D1/D2 sequence and 100% identical for the ITS sequence (639/639 bases matched). Conversely, comparison of R-3610 to the *O. populinus* resulted in insignificant identities for both the ITS and D1/D2 sequences (<90%). Based on these sequence identities, R-3610 was identified as *O. corticola*.

In vitro antifungal susceptibility testing

Antifungal susceptibility tests were performed through the use of a macrobroth dilution method in essential agreement

with that previously published Clinical and Laboratory Standards Institute (CLSI) document M38-A [13]. Amphotericin B (AMB, Bristol-Myers, Squibb, New York, NY) was tested in Antibiotic Medium 3 (Difco, Sparks, MD) while itraconazole (Janssen Pharmaceutica, Piscataway, NJ) was tested in RPMI-1640 (Hardy Diagnostics, Santa Maria, CA). Tubes were incubated at 35°C with minimum inhibitory concentration (MIC) endpoints read at 24 and 48 hours. The endpoints for amphotericin B were the lowest concentrations that inhibited visual growth, while those for itraconazole were 80% inhibition compared to the growth control. Minimum inhibitory concentrations were 0.06/0.5 and 0.06/0.06 µg/ml at 24/48 hours for amphotericin B and itraconazole, respectively.

Discussion

Oxyporus corticola (Fr.) Ryvarden is a white-rot decay fungus of woody angiosperms and gymnosperms and is widely distributed in North America and Europe. It is characterized by resupinate or effuse-reflexed, soft and leathery fruiting bodies with a cream to light brown pore surface. The hyphae are simple, septate with thin to thickened walls, bearing short, clavate basidia on each of which are formed four, ovoid to broadly ellipsoid spores, 5–9 × 3.5–4.5 µm. Two kinds of cylindrical cystidia are present, i.e., one is apically encrusted with hyaline crystals and the other contains a refractive substance [14,15]. The taxonomic synonyms of *O. corticola* are listed by Lowe [16] andV ampola [17].

The number of filamentous basidiomycetes authenticated as invasive human etiologic agents is small. To date the list includes *Schizophyllum commune* [18–26], a *Coprinus* species [27], *Coprinus delicatulus* [28,29], *Coprinopsis cinerea* (formerly *Coprinuscinerea*, anamorph *Hormographiella aspergillata*) [30], and *Inonotus tropicalis* [31,32]. Diseases caused by these fungi are often associated with immune-compromised patients (but also with immune-competent individuals) and range from sinusitis, invasion of the hard and soft palates, pulmonary disease, to brain abscess formation and endocarditis, both of which may be fatal.

The only other basidiomycete to have been previously reported in a dog is *S. commune* which was recovered in Japan from a mongrel dog with a subcutaneous nodule on the neck and lung involvement. Sequencing of the 25S large-subunit ribosomal DNA was required for its identification. This dog died following three months of ketoconzole therapy but was not subject to a necropsy [33].

The isolate from the dog in this report also required molecular characterization for its identification. Filamentous basidiomycetes in culture often fail to form any diagnostic structures on which to base a morphologic identification. Comparison of ITS and D1/D2 sequences with two known isolates of *O. corticola* and an isolate of *O. populinus* provided an unequivocal identification of the etiologic agent. This dog, a GSD presented with a fungal osteomyelitis. Members of this same breed have been reported to be more susceptible to nasal and disseminated fungal aspergillosis with immunodeficiency suspected to be contributory, but not specifically identified with immunologic testing [5]. In another report, no differences were noted in the serum, salivary, and tear IgA concentrations between GSD and non-GSD with or without gastrointestinal disease [34]. However, IgA production by 24-hour duodenal explant cultures was significantly lower in GSD with intestinal disease than in other breeds with the same condition suggesting a deficiency in local IgA secretion. The numbers of lamina propria IgA^+ plasma cells in biopsies were not different between the groups [34]. Serum IgA concentrations were higher when compared to reference values in this dog. These results represent circulating serum IgA and, as stated previously, may not correlate to secretory IgA from epithelial surfaces. Whether a secretory deficiency was present in our GSD is unknown, as neither epithelial secretory concentrations nor other tests for immunodeficiency were performed.

The distribution of the fungus within the animal and the host's reaction to its presence was interesting. The etiologic agent's propensity for growth within the endocardium, myocardium, arterial walls and adventitia was striking. There was little luminal invasion which is more characteristic of zygomycosis and aspergillosis. The amount of inflammation associated with the hyphae was also relatively scant in most vascular locations, with few lymphocytes and plasma cells seen. However, there were exceptions as in the periosteal vessels, where there was a more marked accumulation of inflammatory cells. Whether this muted inflammatory response was a consequence of the recent non-steroidal anti-inflammatory therapy or hypoantigenicity or characteristics of the fungus is not known. Besides the propensity for invasion of blood vessels and bone marrow, endocrine glands (adrenal and thyroid) also seemed particularly vulnerable with more inflammation and necrosis.

The optimal approach to management of infections caused by this fungus remains unknown. There is a paucity of *in vitro* antifungal susceptibility data for clinical basidiomycetes, and limited anecdotal reports employing various treatment regimens. One study looking at 44 human isolates determined that, *in vitro*, the group as a whole exhibited low MICs to amphotericin B, 5-fluorocytosine, fluconazole, itraconazole, voriconazole, and posaconazole [35]. *In vitro,* our isolate appeared susceptible to itraconazole and amphotericin B with 48 hours MICs of 0.06 and 0.5 µg/ml,

respectively. Initially, itraconazole appeared effective in suppressing dissemination with decreased pain and limping, but eventual dissemination to multiple organs occurred. In a case of osteomyelitis in a human patient with chronic granulomatous disease due to *Inonotus tropicalis,* triple antifungal therapy with amphotercin B, voriconazole, and caspofungin suppressed the infection, but failed to eradicate it [32]. Amphotericin B therapy was effective in a child with hard and soft palate invasion by *S. commune* [19].

This case extends the spectrum of filamentous basidiomycetes that may cause diseases in animals and humans, discusses the second basidiomycete reported as an etiologic agent of mycotic infection in dogs, and describes a new agent of canine fungal osteomyelitis with dissemination. *O. corticola* may mimic aspergillosis in histopathologic studies. Definitive identification required molecular characterization, reiterating the utility of these assays for sterile moulds, particularly when sequence data for known isolates is available for comparison.

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Oral candidiasis caused by *Kodamaea ohmeri* in a HIV patient in Chennai, India

Thangam Menon,[1] Monica Herrera,[2] Shankar Periasamy,[1] Veena Palanivelu,[1] Rajasekaran Sikhamani[3] and Brian Wickes[2]

[1]*Department of Microbiology, Dr A. L. Mudaliar Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani, Chennai, India,* [2]*Department of Microbiology & Immunology, University of Texas Health Science Center at San Antonio, TX, USA and* [3]*Government Hospital of Thoracic Medicine, Chennai, India.*

Summary

Kodamaea ohmeri was isolated from a 38-year-old HIV seropositive woman with pseudomembranous oral candidiasis. The isolate was identified by the API 20 C yeast identification system and confirmed by sequence analysis. Antifungal susceptibility testing done by E-test showed that the isolate was susceptible to voriconazole, amphotericin B and caspofungin.

A 38-year-old woman, who was HIV seropositive, presented with pseudomembranous oral candidiasis. Laboratory investigations revealed that her total leucocyte count was 4.6×10^3 mm^{-3} with 66% polymorphs; 24% lymphocytes and 10% monocytes; absolute CD4 count was 111 cells mm^{-3} and % CD4 was 7%. Culture of the oral swab in Sabouraud's dextrose agar grew a creamy-white yeast after 48 h, which was germ-tube negative and did not produce chlamydospores on corn meal agar.

The isolate was grown on CHROMagar Candida (CHROMagar Company, Paris, France), and was found to produce pink-coloured colonies after overnight incubation, which changed to blue after 48 h of incubation (Fig. 1). The isolate was identified as *Kodamaea* (*Pichia*) *ohmeri* by the API 20 C yeast identification system (bioMérieux, Marcy l'Etoile, France) at the Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio. Antifungal susceptibility testing done by E-test (AB Biodisk, Sweden) showed susceptibility to voriconazole, amphotericin B and caspofungin, a dose-dependent response to fluconazole (MIC 16 μg ml^{-1}) and resistance to itraconazole (MIC 1 μg ml^{-1}). The patient had been on oral fluconazole (100 mg daily) for a month when she presented with the oral lesions but was lost to follow up.

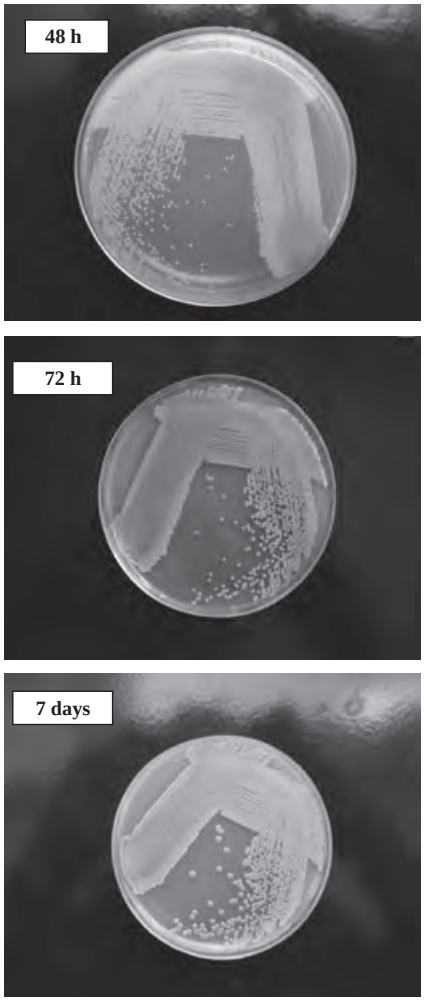

Figure 1 Macroscopic morphology of *Kodamaea ohmeri* grown at 35 °C on CHROMagar *Candida* medium for 48 h, 72 h and 1 week.

Correspondence: Dr Thangam Menon, Department of Microbiology, Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani, Chennai 600 113, Tamil Nadu, India.
Tel.: 91 44 2454 7103. Fax: 91 44 2454 0709.
E-mail: thangam56@gmail.com

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DNA was extracted from the isolate using Prepman Ultra reagent (Applied Biosystems, Foster, CA, USA) and PCR reactions were performed on 3 μl of the Prepman supernatant in a 50 μl reaction using Manual PCR Extender 5 Prime system (5Prime, Gaithersburg, MD, USA) according to the manufacturer's instructions. ITS amplicons were obtained using primers (ITS1 and ITS4) and PCR conditions as previously described (Taylor JW *et al.* PCR-Protocols and Applications-A Laboratory Manual, Academic Press, New York, 1990). D1/D2 PCR amplicons were obtained using primers (NL-1 and NL-4) and PCR conditions as described (Peterson SW and Kurtzman CP, *Antonie Van Leeuwenhoek* 1990: **58**: 235–40; Kurtzman CP and Robnett CJ, *J Clin Microbiol* 1997; **35**: 1216–23). Amplifications were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA, USA), which ran for thirty cycles using an annealing temperature of 60°C and 1 min extension time. A 15 μl aliquot of each PCR reaction was run on a 0.7% agarose gel and stained with ethidium bromide to confirm amplification. The remaining PCR template was prepared for sequencing by cleaning with the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Purified templates were sequenced on both strands at the UTHSCSA Advanced Nucleic Acids Core facility using the same primers for ITS and D1/D2 amplification. Sequencing was performed using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) to prepare sequencing reactions. Sequences were then run on an ABI PRISM 3130xl Genetic Analyzer and data were analysed with Sequencing Analysis Software version 5.3.1 with KB™ Basecaller version1.4 (Applied Biosystems). Sequences were then used to perform individual nucleotide-nucleotide searches using the BLASTn algorithm at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/) and the isolate was identified as *K. ohmeri.*

Kodamaea (*Pichia*) *ohmeri* is the teleomorph of *Candida guilliermondii* var. *membranaefaciens*. It is considered to be an environmental strain, often used in the food industry and rarely seen in human infections (Garcia-Tapia A *et al.*, *Rev Iberoam Mycol* 2007; **24**: 155–6). It has been reported to cause serious infections in immunocompromised patients (Lee JS *et al.*, *J Clin Microbiol* 2007; **45**: 1005–10). Although this teleomorph has been reported in dentures of diabetic and haemodialysed patients (Mendes-Giannini MJ *et al.*, *Mycopathologia* 2007; **164**: 255–63), it has not been reported as a causative agent of oral thrush. The detection of colour change on CHROMagar Candida medium appears to be a definitive and relatively easy method of identifying this organism.

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PLoS GENETICS

Genomic Analysis of the Basal Lineage Fungus *Rhizopus oryzae* Reveals a Whole-Genome Duplication

Li-Jun Ma[1]*, Ashraf S. Ibrahim[2], Christopher Skory[3], Manfred G. Grabherr[1], Gertraud Burger[4], Margi Butler[5], Marek Elias[6], Alexander Idnurm[7], B. Franz Lang[4], Teruo Sone[8], Ayumi Abe[8], Sarah E. Calvo[1], Luis M. Corrochano[9], Reinhard Engels[1], Jianmin Fu[10], Wilhelm Hansberg[11], Jung-Mi Kim[12], Chinnappa D. Kodira[1], Michael J. Koehrsen[1], Bo Liu[12], Diego Miranda-Saavedra[13], Sinead O'Leary[1], Lucila Ortiz-Castellanos[14], Russell Poulter[5], Julio Rodriguez-Romero[9], José Ruiz-Herrera[14], Yao-Qing Shen[4], Qiandong Zeng[1], James Galagan[1], Bruce W. Birren[1], Christina A. Cuomo[1☯], Brian L. Wickes[10☯*]

1 The Broad Institute of MIT and Harvard, Cambridge, Massachusetts, United States of America, **2** Los Angeles Biomedical Research Institute, Harbor-UCLA Medical Center, Torrance, California, United States of America, **3** Bioproducts and Biocatalysis Research, National Center for Agricultural Utilization Research, USDA-ARS, Midwest Area, Peoria, Illinois, United States of America, **4** Department of Biochemistry, Université de Montréal, Montreal, Canada, **5** Department of Biochemistry, University of Otago, Otago, New Zealand, **6** Department of Botany, Faculty of Science, Charles University, Prague, Czech Republic, **7** Division of Cell Biology and Biophysics, School of Biological Sciences, University of Missouri, Kansas City, Missouri, United States of America, **8** Research Faculty of Agriculture, Hokkaido University, Sapporo, Japan, **9** Departamento de Genética, Universidad de Sevilla, Sevilla, Spain, **10** Department of Microbiology and Immunology, University of Texas Health Science Center at San Antonio, San Antonio, Texas, United States of America, **11** Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City, Mexico, **12** Department of Plant Biology, University of California Davis, Davis, California, United States of America, **13** Cambridge Institute for Medical Research, Cambridge, United Kingdom, **14** Departamento de Ingeniería Genética, Unidad Irapuato, Centro de Investigación y de Estudios Avanzados del IPN, Mexico City, Mexico

Abstract

Rhizopus oryzae is the primary cause of mucormycosis, an emerging, life-threatening infection characterized by rapid angioinvasive growth with an overall mortality rate that exceeds 50%. As a representative of the paraphyletic basal group of the fungal kingdom called "zygomycetes," *R. oryzae* is also used as a model to study fungal evolution. Here we report the genome sequence of *R. oryzae* strain 99–880, isolated from a fatal case of mucormycosis. The highly repetitive 45.3 Mb genome assembly contains abundant transposable elements (TEs), comprising approximately 20% of the genome. We predicted 13,895 protein-coding genes not overlapping TEs, many of which are paralogous gene pairs. The order and genomic arrangement of the duplicated gene pairs and their common phylogenetic origin provide evidence for an ancestral whole-genome duplication (WGD) event. The WGD resulted in the duplication of nearly all subunits of the protein complexes associated with respiratory electron transport chains, the V-ATPase, and the ubiquitin–proteasome systems. The WGD, together with recent gene duplications, resulted in the expansion of multiple gene families related to cell growth and signal transduction, as well as secreted aspartic protease and subtilase protein families, which are known fungal virulence factors. The duplication of the ergosterol biosynthetic pathway, especially the major azole target, lanosterol 14α-demethylase (*ERG11*), could contribute to the variable responses of *R. oryzae* to different azole drugs, including voriconazole and posaconazole. Expanded families of cell-wall synthesis enzymes, essential for fungal cell integrity but absent in mammalian hosts, reveal potential targets for novel and *R. oryzae*-specific diagnostic and therapeutic treatments.

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* E-mail: lijun@broad.mit.edu (LJM); wickes@uthscsa.edu (BLW)

☯ These authors are joint senior authors on this work.

Introduction

The fungal kingdom comprises an estimated 1.5 million diverse members spanning over 1 billion years of evolutionary history. Within the fungal kingdom, four major groups ("Phyla")—the Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota—are traditionally recognized [1,2] (Figure 1). Recent phylogenetic studies confirm a monophyletic group (the Dikarya) that includes the ascomycetes and basidiomycetes, and proposed polyphyletic states for the two basal lineages of chytridiomycetes and zygomycetes [3]. The majority of fungal genomic resources generated thus far are for the Dikarya (http://www.ncbi.nlm.nih.gov/genomes/leuks.cgi) and typically focused on fungi that are pathogenic. However, many members of the basal lineages also are important pathogens [4,5] while others serve as outstanding models for understanding the evolution of the entire fungal kingdom. This study reports the analysis of the genome sequence of *Rhizopus oryzae*, which represents the first fungus sequenced from the polyphyletic basal lineages described as the zygomycetes [3].

Author Summary

Rhizopus oryzae is a widely dispersed fungus that can cause fatal infections in people with suppressed immune systems, especially diabetics or organ transplant recipients. Antibiotic therapy alone is rarely curative, particularly in patients with disseminated infection. We sequenced the genome of a pathogenic *R. oryzae* strain and found evidence that the entire genome had been duplicated at some point in its evolution and retained two copies of three extremely sophisticated systems involved in energy generation and utilization. The ancient whole-genome duplication, together with recent gene duplications, has led to the expansion of gene families related to pathogen virulence, fungal-specific cell wall synthesis, and signal transduction, which may contribute to the aggressive and frequently life-threatening growth of this organism. We also identified cell wall synthesis enzymes, essential for fungal cell integrity but absent in mammals, which may present potential targets for developing novel diagnostic and therapeutic treatments. *R. oryzae* represents the first sequenced fungus from the early lineages of the fungal phylogenetic tree, and thus the genome sequence sheds light on the evolution of the entire fungal kingdom.

R. oryzae is a fast growing, filamentous fungus and is by far the most common organism isolated from patients with mucormycosis, a highly destructive and lethal infection in immunocompromised hosts [4,5]. Approximately 60% of all disease manifestation and 90% of all rhinocerebral cases are caused by *R. oryzae* [6]. The rapid growth rate and the angioinvasive nature of the disease leads to an overall mortality of >50% [7]. In the absence of surgical removal of the infected focus, antifungal therapy alone is rarely curative, resulting in 100% mortality rate for patients with disseminated disease [8].

The genus *Rhizopus* was first described in 1821 by Ehrenberg and belongs to the order Mucorales in the phylum Zygomycota [9]. Unlike the Dikarya, fungal species belonging to this basal lineage are characterized, in part, by aseptate hyphae. If septa are produced, they occur only between the junctions of reproductive organs and mycelium, or occasionally between aged mycelia. As a saprobe, *Rhizopus* is ubiquitous in nature and a number of species in the genus are used in industry for food fermentation (e.g., tempeh, ragi), production of hydrolytic enzymes, and manufacture of the fermentation products lactic acid and fumaric acid [10].

There are taxonomic complications within the *Rhizopus* genus, including the recently proposed reclassification of *R. oryzae* (previous synonym *R. arrhizus*) to include two species, *R. oryzae* and *R. delemar* [11]. According to this new nomenclature, the sequenced strain 99–880 would be reclassified as *R. delemar*, but will be referred to as *R. oryzae* in this study in an effort to minimize confusion until this nomenclature is widely accepted.

Analysis of the *R. oryzae* genome provides multiple lines of evidence to support an ancient whole-genome duplication (WGD), which has resulted in the duplication of all protein complexes that constitute the respiratory electron transport chain, the V-ATPase, and the ubiquitin–proteasome system. The ancient WGD, together with recent gene duplications, have led to the expansion (2- to 10-fold increase) of gene families related to pathogen virulence, fungal-specific cell wall synthesis, and signal transduction, providing *R. oryzae* the genetic plasticity that could allow rapid adaptation to adverse environmental conditions, including host immune responses.

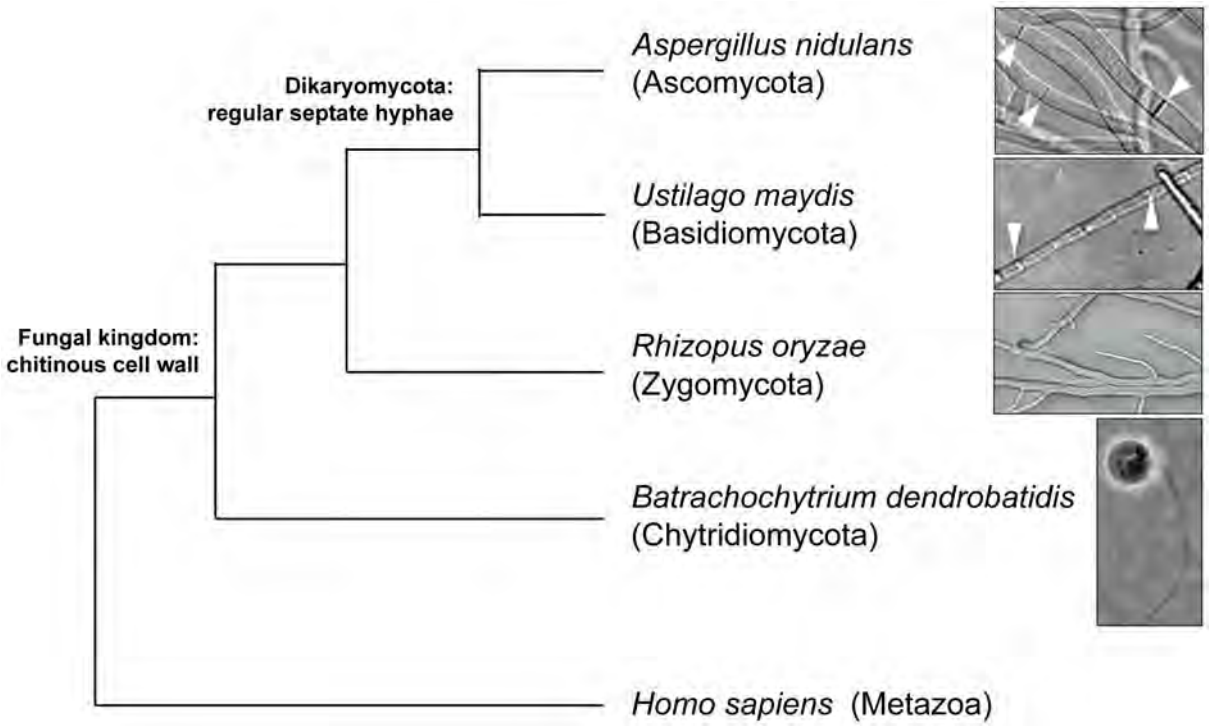

Figure 1. Relationship of major phyla within the fungal kingdom. Phylogeny is shown as a dendrogram using *H. sapiens* (Metazoa) as the out-group. *B. dendrobatidis* (phylum Chytridiomycota) is a unicellular organism with flagellated spores. The terrestrial multicellular fungi include the monophyletic Dikaryomycota (Ascomycota and Basidiomycota) and the more basal fungal lineages, including *R. oryzae*. In contrast to the Dikaryomycota fungi that form hyphae divided by septa (white arrows), the hyphae of *R. oryzae* are multinucleate but not divided into separate cells (coenocytic).
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Results/Discussion

Genome sequencing and organization

Rhizopus oryzae strain 99–880, isolated from a fatal case of mucormycosis, was chosen for whole genome sequencing. The whole genome shotgun reads were generated using Sanger sequencing technology (Materials and Methods, Table S1). The genome assembly consists of 389 sequence contigs with a total length of 45.3 Mb and an N_{50} contig length of 303.7 kilobases (kb) (that is, 50% of all bases are contained in contigs of at least 303.7 kb). Over 11-fold sequence coverage provides high base accuracy within the consensus sequence, with more than 99.5% of the sequence having quality scores of at least 40 (1 error every 10^4 bases) (Table 1).

An *R. oryzae* optical map of 52-fold physical coverage, consisting of 15 linkage groups, was constructed to anchor the assembly and to generate a physical map. The 22 largest scaffolds (44 Mb), corresponding to over 96% of the assembled bases, cover 95% of the optical map (Materials and Methods, Table S2), reflecting the long-range continuity of the assembly and near complete genome coverage. The remaining 5% of the optical map falls into gaps in the assembly or within the highly repetitive ends of linkage groups. We also linked reads containing telomeric tandem repeats $(CCACAA)_n$ to 12 of the 30 linkage group ends, confirming that the assembly extends close to telomeric repeats (Materials and Methods, Figure 2).

Repeat and transposable elements

The *R. oryzae* genome is highly repetitive compared with other fungal genomes (Materials and Methods, Table S3). Over 9 Mb of sequence, accounting for 20% of the assembly, consists of identifiable transposable elements (TEs) (Materials and Methods, Table 2). These include full-length and highly similar copies of many diverse types of TEs from both Class I (retrotransposon) and Class II (DNA transposon) elements. The active transcription of some TEs is supported by the identification of corresponding expressed sequence tags (ESTs) (Materials and Methods, Table 2 and Table S4), suggesting that these elements may be currently active. The Ty3/gypsy-like long terminal repeat (LTR) retrotransposons are the most abundant type of TEs, accounting for 8% of the assembly. The overall distribution of these LTR elements exhibits strong insertion-site preference, often co-localizing with tRNA genes (Figure S1).

Table 1. *Rhizopus oryzae* genome statistics.

Assembly statistics	
Total contig length (Mb)	45.26
Total scaffold length (Mb)	46.09
Average base coverage (Fold)	11
N_{50} contig (kb)	303.66
N_{50} scaffold (Mb)	3.1
Linkage groups	15
GC-content (%)	35.6
Coding (%)	40.6
Non-coding (%)	32.6
Coding sequence	
Percent coding (%)	39.0
Average gene size (bp)	1212
Average gene density (kb/gene)	2.6
Protein-coding genes	17,467
Exons	57,981
Average exon size (bases)	310
Exons/gene	3.3
tRNA genes	239
Non-coding sequence	
Introns	40,514
Introns/gene	2.32
Average intron length (base)	79
Intergenic regions	17,546
Average intergenic distance (bp)	1420

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Genome annotation and evidence for a whole-genome duplication

A total of 17,467 annotated protein-coding genes, including 13,895 genes not overlapping TEs, were predicted in the *R. oryzae* genome (Materials and Methods, Table 1). About 45% of the non-TE proteins have paralogs within the genome and are grouped into 1,870 multi-gene families. Moreover, 17% of these paralogous genes are grouped into two-member gene families, more than two-fold higher than any other representative fungal genome (Materials and Methods, Figure S2). This high proportion of duplicated gene pairs prompted an investigation into whether multiple segmental duplications or an ancestral whole-genome duplication (WGD) event occurred in *R. oryzae*.

WGD was first proposed in *Saccharomyces cerevisiae* based on the order and orientation of duplicated genes in the corresponding chromosomes [12]. This was further confirmed by comparison to a related, non-duplicated species that identified a signature of 457 duplicated gene pairs interleaved with asymmetric gene loss in duplicated regions [13,14]. In the *R. oryzae* genome, we identified 648 paralogous gene pairs, which can be uniquely grouped into 256 duplicated regions containing at least three, and up to nine, duplicated genes (Materials and Methods, Figure S3, and Table S5, S6). Together the duplicated regions cover approximately 12% of the genome and span all 15 linkage groups (Figure 2 and Table S5). The duplicated genes in each of these regions are found in the same order and orientation, providing evidence of an ancestral duplicated state for these regions.

In addition to the similarities of the signature of WGD found in *S. cerevisiae*, we observed multiple lines of evidence to support WGD to the exclusion of independent duplications. First, if the 256 duplicated regions in *R. oryzae* are the cumulative result of multiple segmental duplications, some of the early duplicated regions should also be part of later duplication events. Such regions would be present in the genome as triplets. We estimate that the probability of segments being duplicated two or more times approaches a Poisson distribution, in which 47 triplets would be expected within the 256 duplicated segments. However, we only detected three potential triplet regions ($p<10^{-16}$) (Materials and Methods, Table S5), which refutes the model of multiple segmental duplications. Second, we observed a clear correlation between the presence of TEs and breakpoints within duplicated regions, allowing us to extend the initial duplicated regions in the same orientation into larger blocks that span 23% of the genome (Materials and Methods, Figure 2).

The comparison of protein sets of *R. oryzae* and *Phycomyces blakesleeanus*, a distantly related fungus in the order Mucorales that has been recently sequenced at the Joint Genome Institute (http://genome.jgi-psf.org/Phybl1/Phybl1.home.html), further strengthens the WGD argument. A significant excess of gene duplicates is observed in the *R. oryzae* genome compared with *P. blakesleeanus*

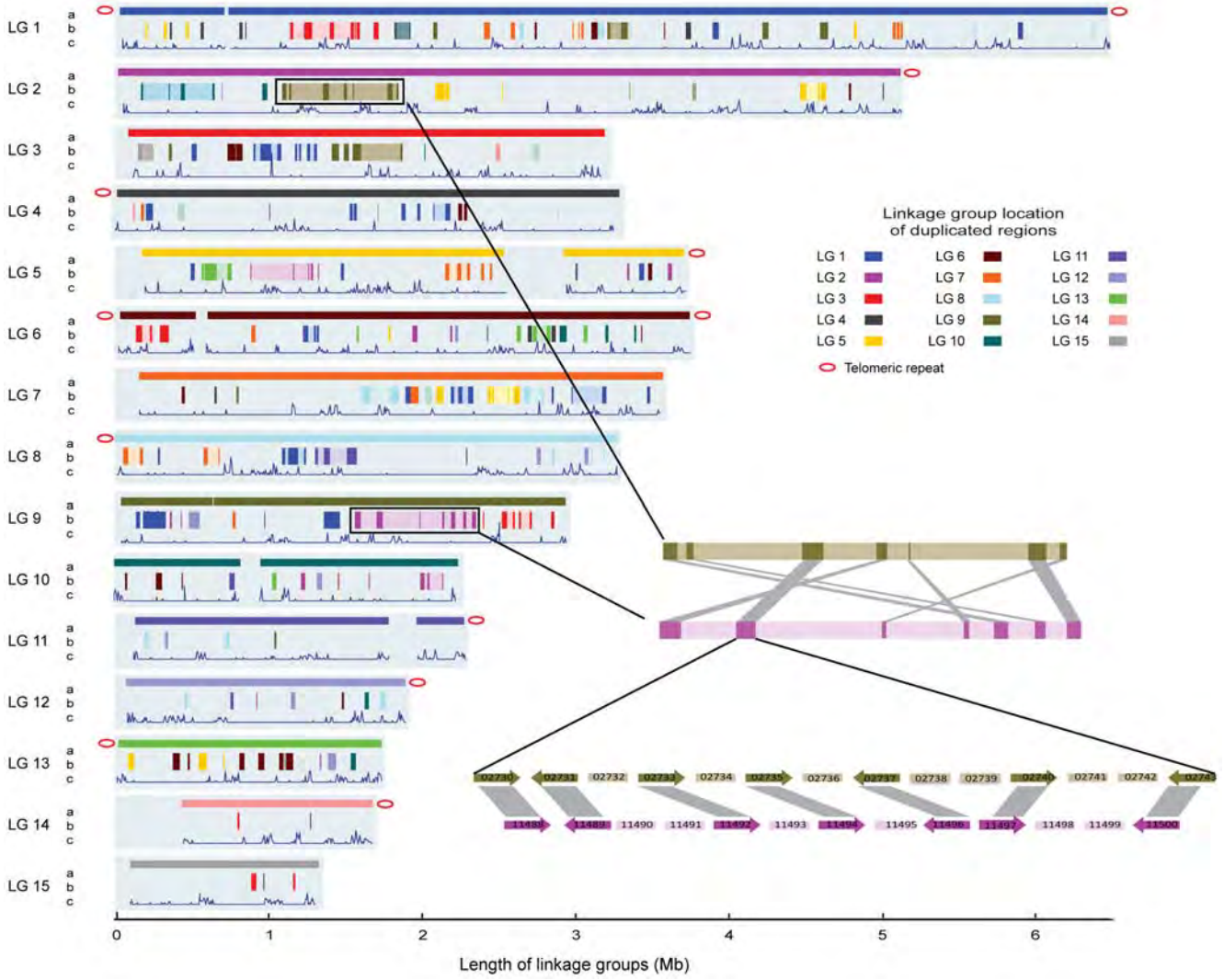

Figure 2. *R. oryzae* genomic structure showing duplicated regions retained after WGD and distribution of LTR transposable elements. The length of the light blue background for each linkage group is defined by the optical map. For each chromosome, row a represents the genomic scaffolds positioned on the optical linkage groups. The red oval indicates linkage to telomeric repeat arrays. Row b displays the 256 duplicated regions capturing 648 gene pairs and spanning 12% of the genome. The shaded backgrounds around some duplicated regions illustrate the duplicated blocks by merging duplicated regions that are within 200 kb after discounting the transposon sequences. These extended duplicated blocks contain the same amount of the duplicates but span 23% of the genome. A pair of corresponding duplicated regions between linkage 2 and linkage 9 are shown in the zoomed images. The numbers in the gene boxes are gene IDs. Row c corresponds to the distribution of the LTR retroelements.
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($p<10^{-16}$) (Materials and Methods, Table S7). Out of the 648 paralogous gene pairs retained in the syntenic regions, 507 share homologs in *P. blakesleeanus* genome. More than 84% (426) of these homologous genes pairs match a single *P. blakesleeanus* gene, reflecting a 2-to-1 correspondence ($p<10^{-150}$). We further estimated the relative duplication time for each duplicated region by averaging the divergences of all the duplicated gene pairs within the region (Figure 3). If the divergence time between *R. oryzae* and *P. blakesleeanus* is defined as t using midpoint rooting (Figure 3A), approximately 78% of all these regions were estimated to be duplicated within one standard deviation (0.115) of the mean ($0.386t$), arguing strongly for a single origin for these duplicated regions (Figure 3B).

Based on the above observations, we conclude that the modern genome of *R. oryzae* arose by a WGD event, followed by massive gene loss. This event resulted in a net gain of at least 648 genes compared to the pre-duplication ancestor. The gene pairs retained after WGD are significantly enriched for protein complexes involved in various metabolic processes (Materials and Methods, Table S8). In particular, we observed the duplication of all protein complexes that constitute the respiratory electron transport chain, the V-ATPase, and the ubiquitin–proteasome systems (Table 3 and Table S9, S10, S11). These protein complexes contain more than 100 protein subunits in total, of which about 80% were retained as duplicates after WGD, including every core subunit of all three complexes. Because an imbalance in the concentration of the subcomponents of large protein–protein complexes can be deleterious [15], duplication of entire complexes should be difficult to achieve by independent duplication events. This observation provides an additional line of evidence to support an ancient WGD in *R. oryzae*.

Large-scale differences exist among the duplicated genes in the post-WGD genomes of *S. cerevisiae* and *R. oryzae*. The increased copy number of some glycolytic genes in *S. cerevisiae* may have conferred a selective advantage in adapting to glucose-rich

Table 2. Transposable elements (TEs) in the *R. oryzae* genome.

Elements	Total bases[a]	% of assembly	Sequence identity (%)[b]	EST[c]
Class I transposons	**5,589,511**	**12.13**		
LTR elements / Ty3	3,700,795	8.03	97%	Yes
LINES	1,742,093	3.78	97%	Yes
DIRS	146,622	0.32	97%	Yes
Class II transposons	**3,462,307**	**7.50**		
Mariners	1,666,728	3.62	98%	Yes
En/Spn	314,481	0.68	98%	No
Tigger	262,307	0.57	94%	No
Crypton	191,823	0.42	98%	No
Helitron	66,534	0.14	99%	No
Total	**9,051,818**	**19.63**		

[a]The genomic distribution of the representative elements was identified using the sensitive mode of RepeatMasker version open-3.0.8, with cross_match version 0.990329.
[b]Sequence identity was computed based on the average identity of the full-length copies of each representative against the consensus sequence of each group.
[c]EST reads overlap with the identified TEs (see Table S6).
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environments through rapid glucose fermentation [16]. The retention of duplicated protein complexes involved in energy generation in *R. oryzae* could have provided an advantage related to the rapid growth of this organism. About 16% of the *R. oryzae* duplicates are also retained in *S. cerevisiae* (BLASTP 1e-5). The genes retained in both systems are enriched for kinases and proteins involved in signal transduction (21%), and proteins involved in transcription/translation processes (21%) (Table S12), possibly indicating potential selective advantage for these genes in both fungal species. Among these shared gene pairs, three out of the four that show accelerated evolution encode enzymatic activities, such as hydrolase, ligase, and protease activities (Table S12).

Gene family expansions

Compared to the genomes of sequenced dikaryotic fungi, several gene families are significantly expanded in *R. oryzae*, including the superclass of P-loop GTPases and their regulators, and the gene families that are essential for protein hydrolytic activities and cell wall synthesis (Materials and Methods, Table 4, and Tables S13, S14, S15, S16).

Expansion of P-loop GTPases and their regulators. To assess the complexity of the basic cellular processes in *R. oryzae*, including proteosynthesis, membrane trafficking, cytoskeletal dynamics, signalling, or cell division, we analyzed in detail a diverse group of proteins central for these processes —the superclass of P-loop GTPases (Table S13) and their regulators (Tables S14). Overall, the general structures of the distinct types of GTPase superclasses and their regulators are very similar in *R. oryzae* compared to dikaryotic fungi. However, a large proportion of these genes have multiple paralogs in *R. oryzae* resulting from gene retention after WGD and additional duplications (Materials and Methods, Table S13). Therefore, the total number of GTPases and their regulators in *R. oryzae* exceeds more than twice and three times, respectively, the number of genes in the other genomes analyzed (Table 4). As the molecular switches that mediate regulatory and signaling steps in diverse cellular processes [17], such an increase might provide the organism an enhanced capacity for coordinating growth and metabolism under highly varied environmental conditions.

Expansion of secreted proteases. The expansion of protease gene families in *R. oryzae* suggests an increased ability of *R. oryzae* to degrade organic matter (Materials and Methods, Table S15) and is consistent with its centuries-old use in fermentation and production of hydrolytic enzymes [10]. The most noteworthy expansions among the protease gene families are of secreted aspartic proteases (SAP) and subtilases (Table 4), which constitute important virulence factors in many pathogenic fungi [18,19]. The large family of *R. oryzae* SAP proteins includes three pairs of genes retained after WGD and three pairs of nearly identical, tandem duplicates that likely arose from recent duplications (Figure S4). The expansion of proteolytic enzymes in *R. oryzae* may facilitate hyphal penetration through decaying organic materials or after establishment of infection through tissues and vessels. Extracellular proteolytic activity of both SAP and subtilase proteins has been linked to virulence in pathogenic *Rhizopus* isolates [20,21], suggesting the potential utility of this group of proteins in vaccine or drug development.

Expansion of fungal cell wall synthesis enzymes. Another important expansion in *R. oryzae* includes gene families that are essential for the biosynthesis of the fungal cell wall, a defining cellular structure that provides physical support and osmotic integrity. Unlike dikaryotic fungi, the cell wall of *R. oryzae* and other Mucorales contains a high percentage of chitin and chitosan, which are synthesized by chitin synthases (CHS) and chitin deacetylases (CDA), respectively [22,23]. The *R. oryzae* CHS and CDA gene families have expanded to 23 and 34 genes, respectively, more than double the numbers observed in any sequenced dikaryotic fungus (Table 4). These families include three pairs of CHS and four pairs of CDA retained after WGD. RT-PCR amplification of the CHS catalytic domains demonstrated that 20 of the 23 CHS, including all the duplicates, are transcribed, suggesting their potential functional roles (Materials and Methods, and Figure 4). Cell wall localization is predicted for 14 of the 34 identified CDA genes based on potential glycosylphosphatidylinositol (GPI)-modification sites (Materials and Methods, Table S16). The surface accessibility of these proteins suggests that they could serve as targets for reliable diagnosis of this invasive pathogen.

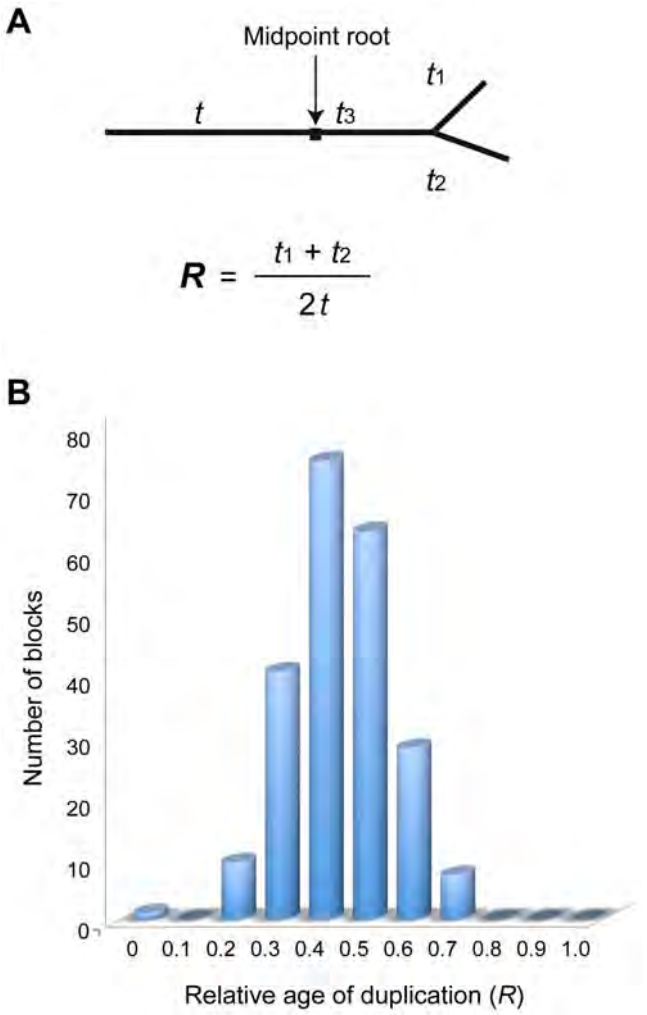

Figure 3. Estimation of duplication dates using *P. blakesleeanus* as an outgroup. (A) An unrooted tree diagram for the duplicated gene pairs in *R. oryzae* and their homologous gene in *P. blakesleeanus*. Midpoint rooting is used to calculate of the relative age of each duplication (*R*) in relation to the root. The branch lengths as substitutions per site for the unrooted tree topology were calculated using the WAG evolutionary model [49] employing a maximum likelihood-based package, PhyML [50]. The distance between two duplicated genes in *R. oryzae* is t_1+t_2, and the distances between the duplicates and their orthologous gene in *P. blaskesleeanus* are $t+t_3+t_1$ and $t+t_3+t_2$, respectively. (B) The distribution of the relative duplication time for each duplicated region in comparison to the root (*R*). *R* is normalized within each duplicated region by averaging the divergences of all the duplicated gene pairs within the region. If the divergence time between *R. oryzae* and *P. blakesleeanus* is defined as *t* using midpoint rooting, approximately 78% of all these regions were estimated to be duplicated within one standard deviation (0.115) of the mean (0.386*t*).
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Ergosterol pathway. The ergosterol biosynthesis pathway is conserved in the *R. oryzae* genome. As a major constituent of the fungal plasma membrane [24], this fungal-specific biosynthetic pathway has been the subject of intensive investigation as a target of antifungal drugs [25]. The conservation of the entire pathway indicates that azoles, a group of drugs that specifically target this pathway [26,27], could be used to treat *R. oryzae* infections. However, about half the genes involved in ergosterol biosynthesis, including the major azole target, lanosterol 14α-demethylase (*ERG11*, RO3G_11790, RO3G_16595), are present in multiple copies (Table S17). Acquisition of azole resistance in a clinical strain of *Candida albicans* reflected amplification of *ERG11* in a gene copy-dependent manner [28,29]. Although experimental validation is pending, the copy number increase and divergence of duplicated protein sequences could contribute to the observed variable responses of *R. oryzae* to different azole drugs, including voriconazole and posaconazole [26,27].

In contrast to the expansions described above, some cell wall synthesis-related genes are underrepresented in the *R. oryzae* genome. For instance, no gene encoding a putative α-1,3-glucan synthase was detected. Compared to four and three copies of β-1,3-glucan synthase (GS) reported in *S. pombe* and *S. cerevisiae*, respectively, the *R. oryzae* genome only contains two GS genes. Nevertheless, the presence of GS underlies the susceptibility of *R. oryzae* to caspofungin acetate, an antifungal agent that inhibits GS [30].

Iron uptake and pathogenicity

Iron is required by virtually all microbial pathogens for growth and virulence [31], and sequestration of serum iron is a major host defense mechanism against *R. oryzae* infection [32]. Genomic analysis reveals that *R. oryzae* lacks genes for non-ribosomal peptide synthetases (NRPSs), the enzymes that produce the most common siderophores (hydroxamate siderophores) used by other microbes to acquire iron. Instead, *R. oryzae* relies solely on Rhizoferrin, which is ineffective in acquiring serum-bound iron [33], and therefore is heavily dependent on free iron for pathogenic growth. This explains why some patients with elevated levels of available free iron, including diabetics, are uniquely susceptible to infection by *R. oryzae* [34]. At the same time, we observed duplication of heme oxygenase (*CaHMX1*) (RO3G_07326 and RO3G_13316), the enzyme required for iron assimilation from hemin in *C. albicans* [35]. Since free iron is usually present at very low concentrations in human blood, the two copies of the heme oxygenase gene may increase iron uptake from host hemoglobin, which would be important for angioinvasive growth. The critical role of iron uptake during *R. oryzae* early infection further reinforces the strategy of treating infections as early as possible with iron chelators that cannot be utilized by *R. oryzae* as a source of iron [36].

Insight into eukaryote evolution

As the first sequenced representative of a fungal lineage basal to the Dikarya, *R. oryzae* provides a novel vantage point for studying fungal and eukaryotic genome evolution. The *R. oryzae* genome shares a higher number of ancestral genes with metazoan genomes than dikaryotic fungi ($p<0.00001$) (Materials and Methods, Table S18). The homologs shared exclusively between *R. oryzae* and Metazoa include genes involved in transcriptional regulation, signal transduction and multicellular organism developmental processes (Figure S5). For example, in contrast to dikaryotic fungi, the *R. oryzae* genome encodes orthologs of the metazoan GTPases Rab32, the Ras-like GTPase Ral, as well as the potential positive regulators of these GTPases (Table S13, S14, Figure S6). The presence of these orthologs suggests that *R. oryzae* might share these metazoan regulatory modules, which are involved in protein trafficking, GTP-dependent exocytosis, and Ras-mediated tumorigenesis [37,38]. In this respect, *R. oryzae* could serve as a model system for studying aspects of eukaryotic biology that cannot be addressed in dikaryotic fungi.

The genome sequence also sheds light on the evolution of multicellularity. As in other Mucorales species, *R. oryzae* hyphae are coenocytic (Figure 1), meaning that the multinucleated cytoplasm is not divided into separate cells by septa after mitosis.

Table 3. Duplication of protein complexes in the *R. oryzae* genome*.

Complexes	Respiratory chain complexes						V-ATPase			Ubiquitin–proteasome system					
Subunits	**I**	**II**	**III**	**IV**	**ATPase**	**Total**	**V_1**	**V_0**	**Total**	**Alpha**	**Beta**	**ATPase**	**LID**	**Modifier**	**Total**
Reference genes	28	4	9	9	10	60	7	5	12	7	7	6	13	3	36
R. oryzae duplicates	20	3	8	8	8	47	5	3	8	6	6	5	10	2	29
% duplicated genes	71.4	75.0	88.9	88.9	80.0	78.3	71.4	60.0	66.7	85.7	85.7	83.3	76.9	66.7	80.6

*Duplicated protein complexes in *R. oryzae* retained after WGD. The reference nuclear genes of protein complexes from *Saccharomyces cerevisiae* or *Neurospora crassa* were used to identify homologous sequences in the *R. oryzae* proteome. We searched for homologous genes using BLASTP (1e–5) and manually checked for short proteins that usually have higher e-values.

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Our analysis suggests that the coenocytic hyphal structure of *R. oryzae* may be attributed to the absence of a functional septation initiation network (SIN), which activates actomyosin ring contraction and the formation of septa upon completion of mitosis [39]. The core components of the SIN pathway, as described in *S. pombe*, and the homologous mitotic exit network (MEN) in *S. cerevisiae*, are common to both fission and budding yeasts (Table S19), including the protein kinases Sid2 (Dbf2p/Dbf20p) and Cdc7 (Cdc15p). Our kinome analysis revealed that *R. oryzae* lacks the Sid2 ortholog. Even though the fungus possesses five copies of Cdc7 homologs, the proteins lack the characteristic C-terminal tail (Figure S7, Table S19). The chytrid fungus *Batrachochytrium dendrobatidis*, fruitfly *Drosophila melanogaster* and nematode *Caenorhabditis elegans* all lack Cdc7 orthologs. This omission suggests that Cdc7 in dikaryotic fungi may have acquired the C-terminal extension, which contributes a significant role in cytokinesis, after the divergence of the lineage leading to *Rhizopus*. Although homologous genes of these two kinase families are also reported in plants and metazoa, their functions are diverged from coordinating the termination of cell division with cytokinesis [40,41]. We therefore hypothesize that the fungal septation pathway may have arisen in the dikaryotic lineage specifically and the multinucleate *R. oryzae* cellular organization may reflect a primitive developmental stage of multicellularity, supporting the theory that multicellularity evolved independently in metazoan, plant, and fungal lineages [42].

Conclusions

Gene duplication plays an important role in genome evolution, thus whole genome duplication (WGD) is expected to have a large impact on the evolution of lineages in which it has occurred [43]. The post-WGD retention of entire protein complexes and gene family expansions could enable *R. oryzae* to rapidly use more complex carbohydrates for energy sources and quickly accommodate major environmental changes. This outcome of WGD may underlie its aggressive disease development observed clinically and its rapid growth rate observed experimentally (Materials and Methods, Table S20).

Due to the lack of suitable laboratory tests, the diagnosis of mucormycosis is notoriously difficult [6]. As an acute and rapidly fatal infection, delayed diagnosis has been associated with a dramatically worse outcome, thus a timely and accurate diagnostic assay is essential for earlier treatment [44]. Our analysis illustrates the value of the *R. oryzae* genome sequence in understanding the basis of angioinvasive pathogenicity and suggests ways to improve diagnosis and treatment. The *R. oryzae* specific cell wall glycoproteins (e.g., the chitin deacetylases) identified through this analysis could serve as targets for reliable diagnosis of this invasive pathogen and therefore could have a profound impact controlling the *R. oryzae* infection.

The *R. oryzae* genome also provides the first glimpse into the genome structure and dynamics of a basal fungal lineage, demonstrating the novel perspective of this model organism for the study of eukaryotic biology that cannot be addressed in

Table 4. Gene family expansion in the *R. oryzae* genome.

Species	Cell wall synthesis		Protein hydrolysis		Cell signaling	
	CHS	**CDA**	**SAP**	**Subtilases**	**GTPases**	**GTPase regulators**
Rhizopus oryzae	**23**	**34**	**28**	**23**	**184**	**246**
Aspergillus fumigatus	9	9	6	4	81	76
Neurospora crassa	7	5	17	8	84	79
Magnaporthe grisea	8	11	8	7	—	—
Saccharomyces cerevisiae	7*	2*	7	4	82	76
Candida albicans	8*	1*	14	2	—	—
Cryptococcus neoformans	8	4	7	2	78	77
Coprinus cinereus	9	16	2	3	86	83
Ustilago maydis	8	8	6	1	80	77

Expanded gene families in *R. oryzae* compared to selected dikaryotic fungal genomes.

—, not tested.

*based on the SGD (http://www.yeastgenome.org/) and CGD (http://www.candidagenome.org/) annotation.

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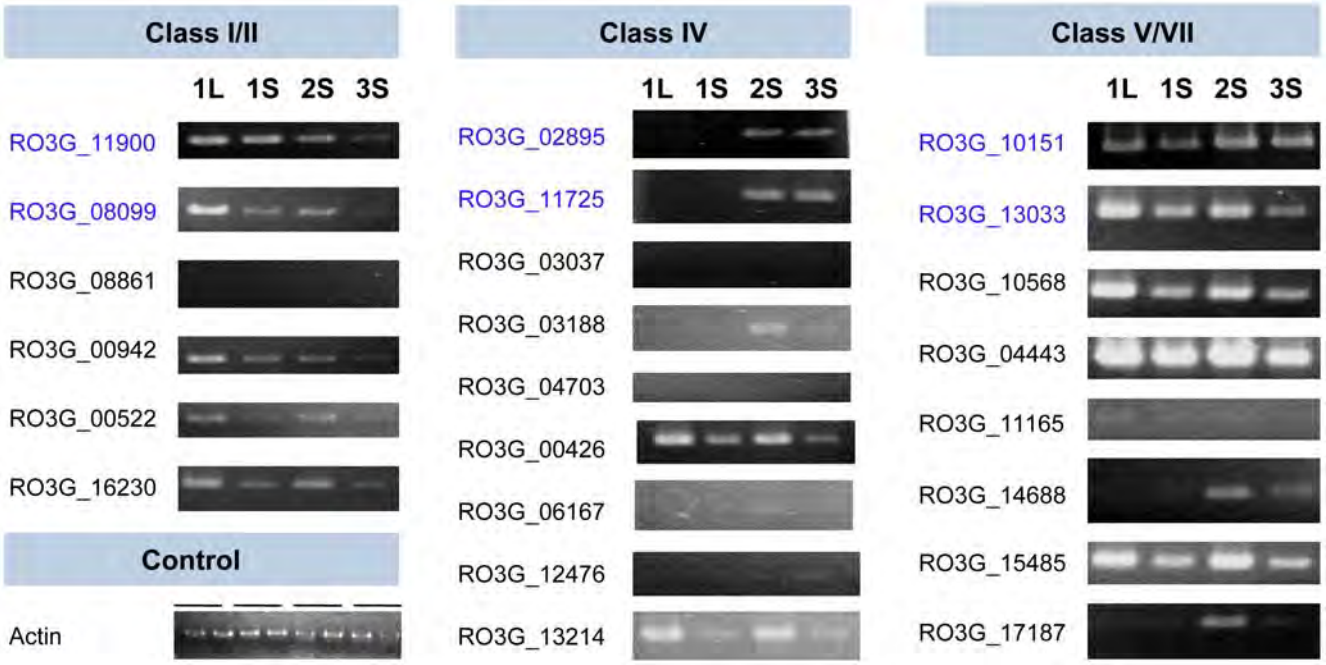

Figure 4. RT–PCR of *R. oryzae* chitin synthases (CHSs). Presence of a transcript was detected from mycelia grown with four different growth phases: 1L, 1-day-old liquid culture; 1S, 1-day-old agar plate; 2S, 2-day-old agar plate; and 3S, 3-day-old agar plate. Gene pairs retained after WGD as detected in the duplicated regions are shown in blue.
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dikaryotic fungi. Importantly, *R. oryzae* gene function can be experimentally studied using transformation [45]. Ongoing sequencing projects for other basal fungi, including two other Mucorales species and at least three chytrids, will further our understanding of the evolution of the fungal kingdom. In addition, the *R. oryzae* sequence also reveals an important observation about the evolution of multicellular eukaryotes, with *R. oryzae* representing a preliminary step toward multicellularity, a trait that evolved multiple times in the history of the different eukaryotic lineages.

Materials and Methods

Sequencing and assembly

Sanger sequencing technology was employed for the *R. oryzae* genome. The sequence was generated using three whole-genome shotgun libraries, including two plasmid libraries containing inserts averaging 4 kb and 10 kb, and a Fosmid library with 40-kb inserts (Table S1), then assembled using Arachne [46].

Optical map

The *R. oryzae* optical map was constructed using restriction enzyme *Bsu*36I [47]. The correspondences of the restriction enzyme cutting sites and the lengths of assembly fragments based on *in silico* restriction were used to order and orient the scaffolds of the assembly to the map (Table S2).

Telomeres

Telomeric tandem repeats $(CCACAA)_n$ of at least 24 bases were identified in the unplaced reads and linked to scaffolds based on read pair information.

Repetitive elements

Repeat sequences were detected by searching the genome sequence against itself using CrossMatch (http://www.genome.washington.edu/UWGC/analysistools/Swat.cfm) and filtering for alignments longer than 200 bp with greater than 60% sequence similarity (Table S3).

Transposable elements (TEs)

The full-length LTR retrotransposons were identified using the LTR_STRUCT program [48]. The DDE DNA transposons were identified using EMBOSS einverted (http://emboss.sourceforge.net/) to locate the inverted repeats, in addition to a BLAST search for the transposase. The LINE elements, DIRS-like elements, Cryptons and Helitrons from *R. oryzae* were detected in a series of TBLASTN searches of the *R. oryzae* sequence database, using the protein sequences as queries. The genomic distribution of the representative elements was identified using the sensitive mode of RepeatMasker version open-3.0.8, with cross_match version 0.990329 (Figure S1).

Gene annotation and gene families

Protein-encoding genes were annotated using a combination of 864 manually curated genes, based on over 16,000 EST BLAST alignments and *ab initio* gene predictions of FGENESH, FGENESH+ and GENEID. Multigene families were constructed by searching each gene against every other gene using BLASTP, requiring matches with $E \leq 10^{-5}$ over 60% of the longer gene length (Figure S2).

Identification of duplicated regions

A duplicated region was defined as two genomic regions that contain at least three pairs of genes in the same order and orientation. The best BLAST hits (2754 gene pairs, among non-TE proteins) with a threshold value of $E \leq 10^{-20}$ were used to search for such duplicated regions. Varying the distance between neighboring gene pairs from 10 kb to 50 kb did not significantly affect the amount of detected duplications (Table S5). We did not

find duplicated regions among sets of genes with randomized locations (1000 permutation tests), attesting to the statistical significance of the duplicated regions detected through this analysis (Figure S3).

If the observed duplicated regions were created through sequential segmental duplications, the duplicated segments will follow a Poisson distribution in the genome.

$$f(x;\lambda)=\frac{\lambda^x\cdot e^{-\lambda}}{x!}$$

where: $e=2.71828$;

x is the probability of which is given by the function; and

λ is a positive real number, equal to the expected number of occurrences that occur during the given interval.

When $f(x;\ 1)=100$; $f(x;\ 2)=18.4$, $f(x;\ 3)=6.13$;

That is, for every 100 duplicates, we expect 18.4 triplications. Thus, for the 256 duplicated regions observed in the *R. oryzae* genome, the expected number of triplications would be 47; however, we only detected three. The probability for this observation is:

$$p(3;47)=\frac{47^3\cdot e^{-47}}{3!}=6.7\times10^{-17}$$

Triplets

All the genes within the duplicated regions, including the non-paralogous genes, were used to compute multiple correspondences with other duplicated regions (Table S8). At a 10-kb distance between neighboring paralogs, we observed 174 duplicated regions, but no triplets, although the expected number of triplets is 32 if duplications were created through sequential segmental duplications. At a 20-kb distance, we only detected three potential triplet regions (Table S5).

Comparative proteomics between *R. oryzae* to *Phycomyces blakesleeanus*

Reciprocal BLAST searches between *P. blakesleeanus* and *R. oryzae* protein sets were conducted using BLASTP, requiring matches with $E\leq10^{-20}$ over 60% of the query gene length (Table S7). For 852 duplicated genes (426 genes pairs) in *R. oryzae*, and their corresponding homologous gene in the *P. blakesleeanus* genome, we constructed unrooted trees (Figure 3A) using PhyML [49]. The mean distance of each gene pair among three homologous genes were calculated using the WAG evolutionary model [50], where the distance between two duplicated genes in *R. oryzae* is t_1+t_2, and the distances between the duplicates and their orthologous gene in *P. blakesleeanus* are $t+t_3+t_1$ and $t+t_3+t_2$, respectively. The relative duplication time of each duplicated region in comparison to the root is calculated as an average duplication time ($R=\frac{1}{2}\ (t_1+t_2)/t$) of all the gene pairs within the region (Figure 3).

Functional enrichment and conservation of retained genes

The non-TE genes were assigned functional annotation using the program Blast2GO [51] (BLAST cut-off = 1e–20). GO term enrichments in the duplicated gene set were determined using Fisher's exact test [52] (Table S8).

Characterization of protein complexes, protein families, and ergosterol pathway

The characterized MRC complex I of *Neurospora crassa* [53] and all other complexes from *Saccharomyces cerevisiae* based on the SGD annotation (http://www.yeastgenome.org/) were used as reference sets to search homologous sequences in the *R. oryzae* proteome (Table S9, S10, S11, S17).

Comparison of P-loop GTPases and their regulators

The GTPases were identified by BLAST and PSI-BLAST searches of the database of predicted *R. oryzae* proteins and the nr database at NCBI using query sequences of major groups of P-loop GTPases and regulators of the Ras superfamily of GTPases culled from the literature. In addition, for identification of proteins containing poorly conserved regulatory domains, HMMER searches were used with HMM profiles built from multiple alignments retrieved from Pfam (http://www.sanger.ac.uk/Software/Pfam/) or SMART (http://smart.embl-heidelberg.de/) collections. Assignment of mutual orthologs is based mainly on reciprocal BLAST (accession numbers of individual GTPases from dikaryotic fungal genomes are available upon request) (Table S13, S14).

Characterization of protein families

Proteolytic enzymes were annotated using HMMER as well as BLAST hits to the Merops peptidase database http://merops.sanger.ac.uk/index.htm; protein numbers from other fungi were downloaded from Merops. BLAST and HMMER (http://hmmer.janelia.org) searches and manual curation were applied to characterize gene families of CHS and CDA (Tables 15). Identification of proteins of probable exocellular locations was determined using Psort algorithms (http://psort.nibb.ac.jp/form2.html) and the presence of a signal peptide (http://www.cbs.dtu.dk/services/SignalP/). The ORFs containing a putative extracellular location and signal peptide were further analyzed for the presence of high levels of serine/threonine residues and high levels of glycosylation using the program at http://us.expasy.org/tools/scanprosite/. The presence of a GPI motif was analyzed with the algorithm located at http://mendel.imp.univie.ac.at/gpi/fungi_server.html.

Growth rate measurement and reverse transcription polymerase chain reaction detection of CHS expression

To compare the growth rate of *R. oryzae* and *A. fumigatus*, the strains were cultured at 37°C with 10^2 spores/5 µl inoculation (Table S20). For RT-PCR tests, *R. oryzae* strain CBS 112.07 was inoculated into a MEB medium or on a MEA plate. RNA was isolated from harvested mycelia using ISOGEN (Nippon Gene, Toyma Japan), followed by purification and treatment with DNase. Detection of each chitin synthase gene transcript was performed using RT-PCR amplification with primers specific to the CHS domain sequence of each gene. Amplification was also performed with RNA that was not treated with reverse transcriptase to serve as a control to determine if the amplification product was from DNA contamination. RT-PCR amplification in a 50 µl reaction mixture with 100 ng of RNA was performed using the QIAGEN One-Step RT-PCR Kit (Valencia, CA). The reaction condition was as follows: reverse transcription at 50°C for 30 min, initial PCR activation step at 95°C for 15 min, 30 cycles of denaturing at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min. A final 10 min of chain elongation at 72°C was carried out after cycle completion in a model 9700 thermal cycler (Applied Biosystems). The reaction condition was as follows: reverse transcription at 50°C for 30 min, initial PCR activation step at 94°C for 2 min, 40 cycles of

denaturing at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 68°C for 2 min. A final 5 min of chain elongation at 68°C was carried out after cycling completion. PCR products were resolved on agarose gels and detected by staining with ethidium bromide (Figure 4).

Comparative proteomics

The protein sets of fungal genomes including *R. oryzae* (non-TE protein set), *Coprinus cinereus*, *Ustilago maydis*, *Fusarium verticillioides*, and *Neurospora crassa* (http://www.broad.mit.edu/annotation/fungi/fgi/), were searched using BLASTP ($E \leq 10^{-20}$) against the NCBI metazoan gene sets (combining the mammal, non-mammalian vertebrates and invertebrates) available at ftp://ftp.ncbi.nlm.nih.gov/gene/DATA/GENE_INFO (February 21, 2008 version) and the dikaryotic database, including the protein sets from Ascomycete fungal genomes (*Aspergillus nidulans*, *Botrytis cinerea*, *Chaetomium globosum*, *Coccidioides immitis*, *Fusarium graminearum*, *Magnaporthe grisea*, *Neurospora crassa*, and *Sclerotinia sclerotiorum*, all generated at the Broad) and the Basidiomycete fungal genomes (*Ustilago maydis*, *Coprinus cinereus*, and *Cryptococcus neoformans* serotype A, generated at the Broad; *Phanerochaete chrysosporium* http://genome.jgi-psf.org/whiterot1/whiterot1.home.html and *Laccaria bicolor* http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html, generated at JGI) (Table S16).

Kinome characterization

A multi-level hidden Markov model (HMM) library of the protein kinase superfamily was applied to the predicted peptides of *R. oryzae* under the HMMER software suite (v. 2.3.2, http://hmmer.janelia.org), correcting for database size with the '-Z' option. The automatically retrieved sequences were individually inspected and protein kinase homologies were determined by building kinase group-specific phylogenetic trees with the annotated kinomes of *S. cerevisiae*, *S. pombe* and *Encephalitozoon cuniculi* [54].

Supporting Information

Figure S1 Co-localization of tRNA genes and some transposable elements.
Found at: doi:10.1371/journal.pgen.1000549.s001 (0.83 MB JPG)

Figure S2 Comparison of protein families among fungal genomes.
Found at: doi:10.1371/journal.pgen.1000549.s002 (0.44 MB JPG)

Figure S3 Distribution of duplicated regions.
Found at: doi:10.1371/journal.pgen.1000549.s003 (0.47 MB JPG)

Figure S4 Phylogeny of fungal secreted aspartyl protease (SAP) proteins.
Found at: doi:10.1371/journal.pgen.1000549.s004 (0.93 MB JPG)

Figure S5 GO annotation of the *R. oryzae* metazoan homologous genes.
Found at: doi:10.1371/journal.pgen.1000549.s005 (0.76 MB JPG)

Figure S6 Maximum-likelihood tree of the RasGEF proteins.
Found at: doi:10.1371/journal.pgen.1000549.s006 (1.25 MB JPG)

Figure S7 The diagram for Cdc15p homologue.
Found at: doi:10.1371/journal.pgen.1000549.s007 (0.45 MB JPG)

Table S1 *Rhizopus oryzae* genome sequence strategy.
Found at: doi:10.1371/journal.pgen.1000549.s008 (0.05 MB PDF)

Table S2 *R. oryzae* assembly mapped to the optical map.
Found at: doi:10.1371/journal.pgen.1000549.s009 (0.04 MB PDF)

Table S3 Repeat content in fungal genomes.
Found at: doi:10.1371/journal.pgen.1000549.s010 (0.05 MB PDF)

Table S4 EST reads corresponding to identified TEs.
Found at: doi:10.1371/journal.pgen.1000549.s011 (0.04 MB PDF)

Table S5 Syntenic blocks for different distance parameters.
Found at: doi:10.1371/journal.pgen.1000549.s012 (0.06 MB PDF)

Table S6 *R. oryzae* syntenic regions and gene pairs that define each region.
Found at: doi:10.1371/journal.pgen.1000549.s013 (0.53 MB PDF)

Table S7 Best-blast hits between *P. blakesleeanus* and *R. oryzae*.
Found at: doi:10.1371/journal.pgen.1000549.s014 (0.07 MB PDF)

Table S8 GO term enrichment among the retained genes (Fisher's exact tests).
Found at: doi:10.1371/journal.pgen.1000549.s015 (0.06 MB PDF)

Table S9 Duplication of oxidative phosphorylation protein complexes.
Found at: doi:10.1371/journal.pgen.1000549.s016 (0.12 MB PDF)

Table S10 Duplication of V-ATPase.
Found at: doi:10.1371/journal.pgen.1000549.s017 (0.07 MB PDF)

Table S11 Duplication of ubiquitin-proteosome system.
Found at: doi:10.1371/journal.pgen.1000549.s018 (0.08 MB PDF)

Table S12 Retained genes in both *R. oryzae* and *S. cerevisiae*.
Found at: doi:10.1371/journal.pgen.1000549.s019 (0.09 MB PDF)

Table S13 Comparison of P-loop GTPases in *R. oryzae* and dikaryotic fungi.
Found at: doi:10.1371/journal.pgen.1000549.s020 (0.21 MB PDF)

Table S14 Regulators of Ras superfamily GTPases in *R. oryzae* and dikaryotic fungi.
Found at: doi:10.1371/journal.pgen.1000549.s021 (0.21 MB PDF)

Table S15 Enriched proteases gene families.
Found at: doi:10.1371/journal.pgen.1000549.s022 (0.08 MB PDF)

Table S16 Annotation of cell wall synthesis enzymes and secreted proteases.
Found at: doi:10.1371/journal.pgen.1000549.s023 (0.06 MB PDF)

Table S17 Ergosterol biosynthesis pathway in *R. oryzae*.
Found at: doi:10.1371/journal.pgen.1000549.s024 (0.06 MB PDF)

Table S18 Fungal homologs to Metazoa.
Found at: doi:10.1371/journal.pgen.1000549.s025 (0.07 MB PDF)

Table S19 Comparison of the core elements of the MEN/SIN pathway.
Found at: doi:10.1371/journal.pgen.1000549.s026 (0.09 MB PDF)

Table S20 Growth comparison (37°C) of *R. oryzae* 99–880 versus *A. fumigatus* AF293.
Found at: doi:10.1371/journal.pgen.1000549.s027 (0.08 MB PDF)

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Batrachochytrium dendrobatidis zoospore image was provided by Joyce Longcore at the University of Maine.

Author Contributions

Conceived and designed the experiments: LJM CS BWB BLW. Performed the experiments: LJM BFL TS AA JF. Analyzed the data: LJM ASI MGG GB MB ME AI TS SEC LMC WH JMK BL DMS LOC RP JRR JRH YQS CAC. Contributed reagents/materials/analysis tools: ASI CS MGG BFL RE CDK MJK SO QZ JG BLW. Wrote the paper: LJM ASI CS ME AI BL CAC BLW.

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Trichosporon mycotoxinivorans, a Novel Respiratory Pathogen in Patients with Cystic Fibrosis▽

Patrick W. Hickey,[1,2]* Deanna A. Sutton,[3] Annette W. Fothergill,[3] Michael G. Rinaldi,[3] Brian L. Wickes,[4] Howard J. Schmidt,[5,6] and Thomas J. Walsh[7]

Division of Pediatric Infectious Disease[1] and Division of Pediatric Pulmonology,[5] Walter Reed Army Medical Center, Washington, DC; Departments of Pediatrics and Preventive Medicine & Biometrics, Uniformed Services University, Bethesda, Maryland[2]; Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center, San Antonio, Texas[3]; Department of Microbiology, University of Texas Health Science Center, San Antonio, Texas[4]; Division of Pediatric Pulmonology, Virginia Commonwealth University, Richmond, Virginia[6]; and National Cancer Institute, Pediatric Oncology Branch, Immunocompromised Host Section, Bethesda, Maryland[7]

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This report describes the molecular epidemiology, in vitro susceptibility, colonial and microscopic morphologies, and biochemical features of *Trichosporon mycotoxinivorans*, a newly recognized pathogen that appears to have a propensity for patients with cystic fibrosis. The index patient died with histologically documented *Trichosporon* pneumonia complicating cystic fibrosis. This is also the first report of disease caused by a *Trichosporon* species in a nontransplant patient with cystic fibrosis. As *T. mycotoxinivorans* has not previously been recognized as a respiratory pathogen, the significance of its recovery from sputum samples was not initially appreciated. Genetic analysis of archived clinical samples found three additional cases of *T. mycotoxinivorans* infection which had previously been identified as other members of the genus. An additional isolate of *T. mycotoxinivorans* was identified from a clinical sample on initial testing. Three of these four cases were also patients with cystic fibrosis. All isolates had MICs at 48 h of amphotericin B of ≥1 μg/ml and of echinocandins of ≥16 μg/ml, but they displayed various susceptibilities to the triazoles. In summary, *Trichosporon mycotoxinivorans* is a newly recognized human pathogen that is associated with cystic fibrosis.

Trichosporon species are uncommon but potentially life-threatening causes of localized and disseminated infections in immunocompromised patients. The most common species causing deep-seeded disseminated infection that has been recognized is *Trichosporon asahii* (11). Other species of *Trichosporon* are seldom reported as causes of deep-seeded infections. Herein, we describe the first case of human disease caused by *Trichosporon mycotoxinivorans*. Previously recognized as an industrial source for detoxification of mycotoxins, *T. mycotoxinivorans* has not been reported to cause disease in humans (20, 28). Additional testing of archived isolates of the genus *Trichosporon* at the University of Texas San Antonio Fungus Testing Laboratory (FTL) revealed three additional cases, including two in patients with cystic fibrosis. These isolates had previously been classified under other species of *Trichosporon* on the basis of their biochemical profiles alone. A newly acquired sample in the FTL collection, from a cystic fibrosis patient, was also analyzed and identified as *T. mycotoxinivorans.* This report describes the molecular epidemiology, in vitro susceptibility, colonial and microscopic morphologies, and biochemical features of *T. mycotoxinivorans*, a newly recognized pathogen that appears to have a propensity for patients with cystic fibrosis.

Index case. A 20-year-old male with a history of cystic fibrosis presented to the emergency department complaining of acute onset of dyspnea. He was found to have a right-sided pneumothorax. He was treated with supplemental oxygen, a chest tube was placed, and he was then admitted to the ward for supportive care. His recent medical history was notable for receiving home therapy with piperacillin-tazobactam and trimethoprim-sulfamethoxazole, initiated 1 week earlier as an inpatient for an exacerbation of his underlying chronic lung infection from cystic fibrosis. His most recent preadmission sputum cultures were positive for methicillin-resistant *Staphylococcus aureus* and *Candida albicans.* His history showed that a sputum sample had grown multidrug-resistant *Burkholderia cepacia.* His chronic medications included nebulized tobramycin, nebulized dornase alpha, nebulized DNase (pulmozyme), oral pancrelipase, oral azithromycin, and inhaled fluticasone propionate (250 μg) plus salmeterol (50 μg) as one inhalation twice daily. During the year leading up to this admission his forced expiratory volume in the first second (FEV1) had declined from a baseline of 58% of that predicted (14) to 47% of that predicted at 3 months prior to admission and to 24% of that predicted at the most recent admission. Due to the severe drop in FEV1, in addition to antibiotics, he was treated with prednisone at 80 mg once daily for 3 days leading up to the final admission. The patient's nutritional status had been stable but compromised in recent years, and his body mass index was less than the third percentile for his age.

Initial management for the readmission focused on pul-

* Corresponding author. Mailing address: Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd., Bethesda, MD 20814-4712. Phone: (301) 295-9779. Fax: (301) 295-1971. E-mail: phickey@usuhs.mil.

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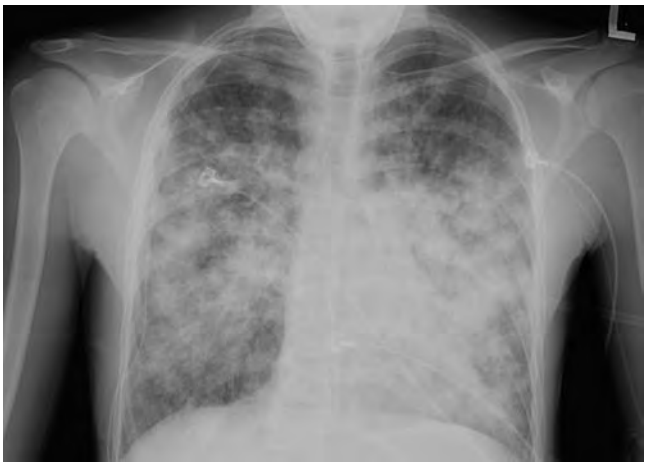

FIG. 1. Hospital day 3 chest X ray showing residual right-sided pneumothorax and bilateral pneumonia.

TABLE 1. Respiratory microbiology results for index case

Hospital day	Organism(s) isolated[a]	Source
1	4+ *Trichosporon*	Sputum
2	4+ *Trichosporon*, 2+ MRSA	Sputum
2	Influenza virus/parainfluenza virus/ adenovirus/respiratory syncytial virus culture negative	Nasopharyngeal wash
3	4+ *Trichosporon*	Bronchoalveolar lavage
5	4+ *Trichosporon*	Tracheal aspirate
6	4+ *Trichosporon*, 3+ *C. albicans*	Tracheal aspirate
10	4+ *Trichosporon*, 3+ MRSA	Tracheal aspirate
10	AFB negative	Tracheal aspirate
11	4+ *Trichosporon*, 3+ MRSA	Tracheal aspirate
12	3+ *Trichosporon*	Right lower lung, autopsy
12	4+ *Trichosporon*	Left lower lung, autopsy
12	4+ *Trichosporon*	Right middle lung, autopsy
12	Negative bacterial, AFB, and viral cultures	All lung fields, autopsy

[a] MRSA, methicillin-resistant *Staphylococcus aureus*; AFB, acid-fast bacillus; 2+, >10 colonies observed in quadrant 1 of the culture plate; 3+, >10 colonies observed in quadrant 2 of the culture plate; 4+, >10 colonies observed in quadrant 3 of the culture plate.

monary toilet, and his medications were not changed. His pulmonary function worsened over the next 2 days, as evidenced by increasing supplementary oxygen requirements, and he was transferred to the pediatric intensive care unit. Vancomycin and intravenous tobramycin were added to the regimen, and trimethoprim-sulfamethoxazole was discontinued. Chest radiographs during this time worsened from the small residual right-sided pneumothorax status noted after placement of the chest tube to infiltrates in the right middle and right lower lobes and the left lower lung fields (Fig. 1). On the following day, his condition worsened and he required intubation. He subsequently became febrile and had multiple fevers each day, with his temperatures ranging from 38.3°C to 40°C. Gram stains of sputum from the time of admission were observed by the use of a high-powered microscope under conditions of oil immersion (Fig. 2) and showed no bacteria and >30 budding yeasts and >30 polymorphonuclear leukocytes per field. The microbiology culture results from the respiratory tract over the course of the hospitalization are summarized in Table 1.

Liposomal amphotericin B at a dosage of 5 mg/kg of body weight/day was initiated on hospital day 4. On hospital day 6, the yeast was identified as a *Trichosporon* species on the basis of microscopic morphology and the biochemical testing results obtained with the API 20 C AUX system (API code, 6747776). Isolates grown on cornmeal agar consisted of dry white colonies that microscopically demonstrated true hyphae and consecutive arthroconidia (Fig. 3). "Hockey stick" arthroconidia suggestive of *Geotrichum* species were not seen. Fusiform giant cells also were not observed. The liposomal amphotericin B therapy was stopped, and voriconazole was started due to concerns of intrinsic drug resistance. His course in the intensive care unit was further complicated by tension pneumothorax and cardiac arrest. Because a poor functional outcome was predicted by serial neurological examinations, imaging of the central nervous system, and electroencephalography findings, the family withdrew medical support on hospital day 11. A partial autopsy was performed and revealed diffuse hemorrhagic and suppurative consolidation of the lungs with chronic bronchiectatic changes (Fig. 4). Cultures of tissue from each lobe collected postmortem grew *Trichosporon* species, and histology demonstrated diffuse infiltration of the lung parenchyma with budding yeasts (Fig. 5). A reference laboratory identified the organism as *Trichosporon loubieri* on the basis of

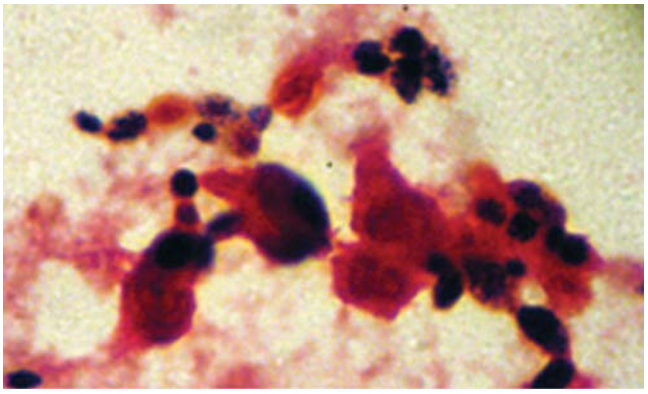

FIG. 2. Gram stain of sputum showing budding yeast and polymorphonuclear cells.

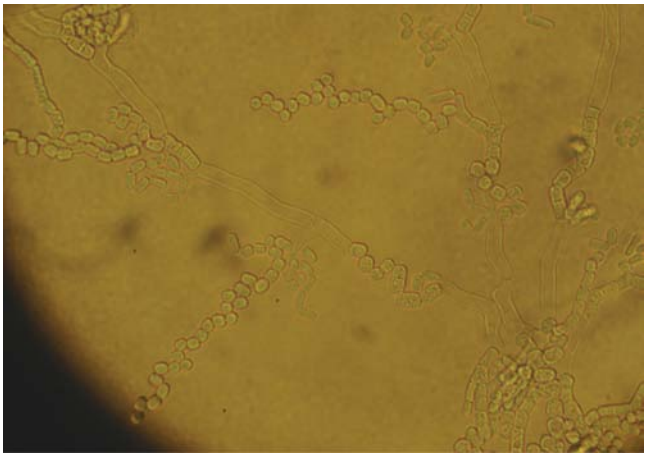

FIG. 3. *Trichosporon* species on cornmeal agar.

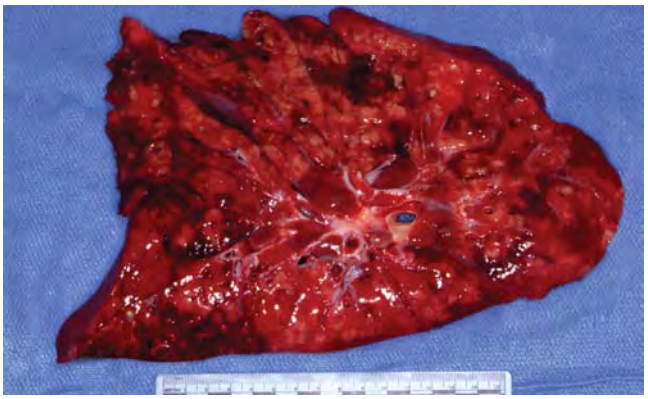

FIG. 4. Postmortem specimen of lung showing diffuse hemorrhagic and suppurative consolidation with bronchiectatic changes suggestive of an acute infection superimposed on a chronic infection and cystic fibrosis-related changes.

morphology and growth characteristics. Additional cultures found no evidence of acid-fast organisms, respiratory viruses, or bacteria. A PCR for human immunodeficiency virus RNA was negative.

MATERIALS AND METHODS

Patients and specimens. The same case isolate originally identified as *T. loubieri* was subsequently referred to FTL, Department of Pathology, University of Texas Health Science Center at San Antonio, for further characterization and was accessioned into its culture collection as UTHSC R-3912. Five archived clinical isolates of the genus *Trichosporon* which had been previously categorized as other members of the genus on the basis of their biochemical profiles and a subsequently acquired sample in the FTL collection were also recovered and analyzed.

Morphological identification. Routine identification of isolates consisted of determination of the assimilation patterns by using the API 20C yeast identification system (bioMérieux, St. Louis, MO) and determination of microscopic morphology on cornmeal agar (Difco, Sparks, MD), temperature tolerance, and susceptibility to cycloheximide (Mycobiotic agar; Remel, Lenexa, KS).

Molecular identification. All cultures were grown and maintained on YPD agar (2% dextrose, 2% peptone, 1% yeast extract, 2% agar) until they were used for analysis. Approximately 1×10^6 yeast cells were obtained from a YPD agar plate grown for 24 h at 30°C and inoculated into 50 μl of Prepman Ultra reagent (Applied Biosystems, Inc., Foster City, CA). The cells were then lysed, according to the manufacturer's instructions. PCR was performed in a 0.5-ml microcentrifuge tube with 5 μl of the Prepman supernatant. A 50-μl reaction volume was prepared with Triple Master *Taq* DNA polymerase (Eppendorf/Brinkmann Instruments, Inc., Westbury, NY), according to the manufacturer's instructions. Two primer pairs were used to amplify two regions of the ribosomal repeat from the genomic DNA template. The internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) was amplified by using primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), as described previously (33) The D1/D2 region of the large ribosomal DNA subunit was amplified with primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3), as described previously (17). All PCRs were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA) by using the preprogrammed three-step protocol as the standard program for all reactions. The annealing temperature for both primer pairs was 58°C, and 30 cycles with a 1-min extension time were used to prepare the amplicons. The PCR products were electrophoresed through a 0.7% agarose gel to confirm amplification. The remaining template DNA was then cleaned with a QIAquick PCR purification kit (Qiagen, Inc., Valencia, CA). Sequencing of both strands was performed at the University of Texas Health Science Center at San Antonio Advanced Nucleic Acids Core Facility with all four PCR primers as the sequencing primers. Both the ITS and the D1/D2 sequences were used to perform nucleotide-nucleotide searches by use of the BLASTn algorithm at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Identifications were made when the BLAST searches yielded ≥98% identity.

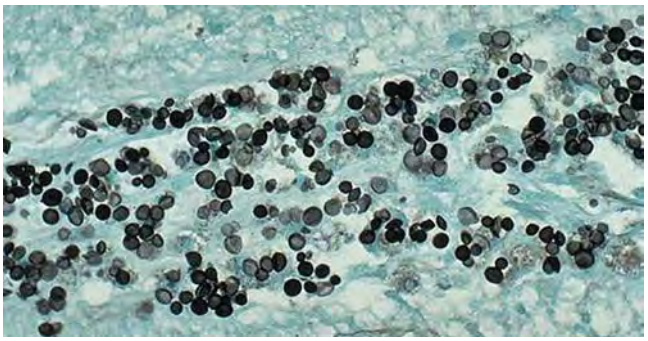

FIG. 5. Postmortem lung biopsy specimen showing fungal elements. Gomori-methenamine silver stain. Magnification, ×100.

In vitro susceptibility studies. Antifungal susceptibility testing was accomplished by the method outlined in NCCLS document M27-A2 (23). Amphotericin B (Bristol-Myers Squibb, New York, NY), caspofungin (Merck, Rahway, NJ), anidulafungin (Pfizer, Inc., New York, NY), and micafungin (Astellas, Deerfield, IL) were tested in antibiotic medium 3 (Difco), while flucytosine (Valient, Irvine, CA), fluconazole (Pfizer, Inc.), itraconazole and ketoconazole (Janssen Pharmaceutica, Piscataway, NJ), posaconazole (Schering Plough, Galloping Hill, NJ), and voriconazole (Pfizer, Inc.) were tested in RPMI 1640 medium (Hardy Diagnostics, Santa Maria, CA). The microtiter plates were incubated at 35°C, and the MICs were read at both 24 and 48 h. The endpoint for amphotericin and the echinocandins was the lowest concentration that inhibited visual growth, while the endpoint for flucytosine and the azoles was the lowest concentration that resulted in a 50% or greater reduction in turbidity compared to that of the drug-free control tube.

Nucleotide sequence accession numbers. The ITS and the D1/D2 sequences were submitted to GenBank under accession numbers EU118973 to EU118984 and FJ416595 to FJ416596, respectively.

RESULTS

Morphological identification. The morphological and molecular identities of seven isolates from five patients are summarized in Table 2 and are most consistent with *T. mycotoxinivorans*. All isolates assimilated glucose, glycerol, 2-keto-D-gluconate, arabinose, xylose, galactose, inositol, methyl-D-glucoside, *N*-acetyl-glucosamine, maltose, sucrose, trehalose, and raffinose. All but one isolate assimilated lactose, and one isolate assimilated cellobiose. All isolates were resistant to cycloheximide, as evidenced by growth on mycobiotic agar. The case isolate has been deposited in the University of Alberta Mold Herbarium, Edmonton, Alberta, Canada, and is accessioned as UAMH 10833.

Molecular identification. The BLASTn search results suggested that the isolates were most closely related to *Trichosporon mycotoxinivorans* (Table 2). The ITS sequences of all isolates were most closely related to the ITS sequences of two *Trichosporon mycotoxinivorans* isolates (strains HB 1175 and TU-GM8, GenBank accession nos. AJ601389 and DQ325457, respectively) at 99% identity and an isolate of *T. loubieri* (strain CBS 7065, GenBank accession no. AB018027) at 98% identity. The D1/D2 sequences of the isolates were most closely related to the D1/D2 sequence of an isolate of *T. mycotoxinivorans* (strains HB 1175, GenBank accession no. AJ601388) at 99% identity and two isolates of *T. loubieri* (strains CBS 7065 and IFM 53857, GenBank accession nos. AF075522 and AB186489, respectively) at 98% identity. Strain HB 1175 is the type culture from which the original description of the species *T. mycotoxinivorans* was described (20).

TABLE 2. Morphological and molecular identification of seven isolates from five patients

Patient no.	FTL no.	GenBank accession no.	API code	Mycosel result	Growth at 42°C	Molecular identification
1[a,b]	R-3912	EU118979	6745776	Pos[c]	Pos	*T. mycotoxinivorans*
2[b]	07-301	EU118980	6747776	Pos	Pos	*T. mycotoxinivorans*
2[b]	07-302	EU118981	6745776	Pos	Pos	*T. mycotoxinivorans*
2[b]	07-303	EU118982	6745376	Pos	Neg[d]	*T. mycotoxinivorans*
3	07-384	EU118983	6745376	Pos	Weak[e]	*T. mycotoxinivorans*
4[b]	07-497	EU118984	6747776	Pos	Weak	*T. mycotoxinivorans*
5[b]	08-288	FJ416596	6747776	Pos	Pos	*T. mycotoxinivorans*

[a] From a patient with cystic fibrosis.
[b] From the index case patient.
[c] Pos, positive.
[d] Neg, negative.
[e] Weak, weakly positive.

In vitro susceptibility. Retrospective in vitro antifungal susceptibility testing data for the index case isolate and the six additional strains identified as *T. mycotoxinivorans* against 10 antifungal agents are displayed in Table 3. The MICs at 48 h of amphotericin B ranged from 1 to 4 μg/ml, while those for the echinocandins caspofungin, micafungin, and anidulafungin were ≥16 μg/ml; but the isolates displayed various susceptibilities to the triazoles.

DISCUSSION

The case described here represents the first reported case of *T. mycotoxinivorans* disease in a human and is the first case of *Trichosporon* species pneumonia in a nontransplant cystic fibrosis patient. *Trichosporon* is a known cause of pneumonia in immunocompromised patients (32). This patient had histologically documented *Trichosporon* pneumonia from postmortem examination, hence fulfilling the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group criteria for proven invasive fungal infection (6). That the isolate was initially identified as *T. loubieri* prompted further investigation to include the genetic analysis, which revealed its true identity. A review of the archives of FTL and five isolates from clinical specimens most closely resembling *Trichosporon ovoides* but with some significant variations in morphology and physiologic features unexpectedly revealed that all were *T. mycotoxinivorans* when they were characterized by ITS and D1/D2 sequencing. Because only a single IGS1 (intergenic spacer 1 region) GenBank record existed for both *T. mycotoxinivorans* and *T. loubieri*, we considered that the use of the more common ITS and D1/D2 regions would be more accurate. Although the match cutoff was >98% identity in order for a sequence to be considered conspecific and two species in the ITS and D1/D2 outputs that met this criterion were found (*T. mycotoxinivorans* and *T. loubieri*), there were multiple matches and they were consistently divided by species, with the *T. mycotoxinivorans* records consistently showing the higher degree of identity (99% versus 98%). Importantly, the sequences from the type culture also showed a higher level of identity correlating with the sequences from the isolates described in this report for both loci.

Four of these isolates (three from the same patient, each of which displayed different macroscopic morphologies) were from cystic fibrosis patients. An additional isolate from a cystic fibrosis patient, analyzed for the first time, was also identified as *T. mycotoxinivorans.* Clinical information from these three patients with cystic fibrosis was not available for review. A comment on *T. loubieri* bears note. *T. loubieri* is an uncommon pathogen that has previously been reported as a cause of human disease in only two other cases. The first case, reported by Padhye et al., describes a 45-year-old man with a history of polycystic kidney disease and end-stage renal disease (24). Cultures of tissue from the left kidney following nephrectomy were performed, and the cultures grew *T. loubieri*. He was

TABLE 3. MIC and time of MIC calculation

FTL no.	MIC (μg/ml) at the indicated times (h) of MIC calculation[c]																			
	AMB		CAS		MICA		ANID		5FC		FLU		ITRA		KETO		VORI		POSA	
	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48
R-3912[a,b]	0.25	1	16	16	>16	>16	>16	>16	4	8	0.5	0.5	0.03	0.06	0.25	0.5	0.03	0.03	<0.015	<0.015
07-301[b]	0.5	4	16	>16	>16	>16	>16	>16	32	>64	64	>64	0.125	1	2	8	>8	>8	0.25	0.5
07-302[b]	0.25	1	16	16	>16	>16	>16	>16	8	32	32	32	0.125	1	4	8	4	8	0.25	1
07-303[b]	0.5	2	8	16	>16	>16	16	16	16	64	64	64	0.03	0.06	0.125	0.5	2	2	0.03	0.06
07-384	0.5	2	16	16	>16	>16	>16	>16	16	32	1	1	0.06	0.06	0.25	0.25	0.06	0.06	<0.015	<0.015
07-497[b]	0.5	2	16	>16	>16	>16	>16	>16	16	64	2	2	0.06	0.5	0.25	0.5	0.06	0.125	<0.015	0.125
08-2888[b]	0.125	1	16	>16	>16	>16	>16	>16	8	32	0.5	2	0.06	0.25	0.5	0.5	0.03	0.06	≤0.015	0.125

[a] From a patient with cystic fibrosis.
[b] From the index case patient.
[c] AMB, amphotericin B, CAS, caspofungin; MICA, micafungin; ANID, anidulafungin; 5-FC, fluconazole; FLU, flucytosine; ITRA, itraconazole; KETO, ketoconazole; VORI, voriconazole; POSA, posaconazole.

successfully treated with a combination of amphotericin B and oral itraconazole. The second case, reported by Marty et al., describes a 56-year-old woman with pre-B-cell lymphoblastic leukemia who developed disseminated *T. loubieri* infection following induction chemotherapy (19). She was treated with oral fluconazole; however, she was unable to proceed with consolidation chemotherapy and soon died. Identification of the organism in both cases was confirmed by ribosomal DNA analysis. Mention of the isolation of *T. loubieri* from the sputum of a cystic fibrosis patient, with unclear clinical relevance, was made in the *Newsletter of the Australasian Federation of Medical and Veterinary Mycology*, but no further clinical details were provided (34). The rarity of this organism and its potential association with other cystic fibrosis patients prompted the more detailed investigation described here.

The taxonomy of the genus *Trichosporon* underwent a significant revision in 1992 on the basis of the molecular analysis of reference isolates, resulting in a schema that included six species, *T. asahii*, *T. mucoides*, *T. inkin*, *T. ovoides*, *T. cutaneum*, and *T. asteroides*, believed to be medically relevant (12). The first two species in this list were grouped into the ecological niche of isolates causing systemic human infections, while the last four species were given the designation of isolates causing superficial infections. Invasive *Trichosporon* species, predominantly known in earlier literature as *T. beigelli*, have most commonly been identified as causes of disease in immunocompromised patients receiving chemotherapy or chronic systemic corticosteroids; they have also occasionally been isolated from burn patients and premature neonates. As reflected by more recently published reports, the preponderance of these isolates likely represent *T. asahii* and *T. mucoides*, which are associated with invasive disease in immunocompromised and, rarely, immunocompetent patients (2, 7, 13, 21). In contrast, *T. ovoides*, *T. cutaneum*, *T. inkin*, and *T. asteroides* (now called *T. jirovici*) are more commonly associated with superficial infections such as white piedra of the scalp or pubic hair (*T. inkin* and *T. ovoides*) and dermatophytic infections (*T. inkin* and *T. jirovici*) and are only rarely associated with central venous line infections or disseminated disease in the immunocompromised host (5, 16). *T. pullulans* bears morphological similarities to the other *Trichosporon* species; however, it is phylogenetically distinct and should be classified elsewhere. Furthermore, some experts believe that the inability to grow at temperatures greater than 20°C functionally excludes its role as a pathogen and that reported cases (18, 22, 29) of *T. pullulans* disease are in fact caused by other species that were misidentified due to a reliance on morphology and biochemical testing alone and not genetic testing (3).

Trichosporon mycotoxinivorans was first described in 2004 by Molnar et al. after isolation of the yeast from the hind gut of a termite (20). Genetic analysis revealed that its ITS sequence had 99.3% homology with the ITS sequence of *T. loubieri*; however, for the IGS1 sequences, the degree of homology was 66.7%. Further study of this yeast revealed that it has the ability to detoxify mycotoxins, such as ochratoxin A and zearalenone, hence the species name, which derives from Latin and which means "mycotoxin devouring." Molnar et al. speculated that this ability would have application in veterinary feeds as a way of detoxifying contaminating mycotoxins (20). This hypothesis was tested by challenging chicks fed a diet containing *T. mycotoxinivorans* with ochratoxin A. Those chicks avoided the immunosuppressive effects experienced by the chicks in the control group and had an improved feed-to-weight gain ratio (25). In vitro studies with a pig intestine model and ochratoxin A challenge demonstrated that treatment with *T. mycotoxinivorans* resulted in the clearance of mycotoxin at a rate comparable to or faster than that of the bacterial comparators *Stenotrophomonas* sp. (90% deactivation) and *Eubacterium* sp. (70% deactivation) at 6 h of incubation (28).

While molecular diagnostic methods, as detailed above, are able to accurately delineate the taxonomic identity of this organism, the availability and timeliness of these resources are limited.

For clinical microbiologists, growth characteristics and microscopic features can help suggest the identity. On glucose-yeast extract-peptone medium, *T. mycotoxinivorans* forms cream to tan mucoid colonies with few white aerial mycelia (20). It forms true hyphae which disarticulate into arthroconidia. Cells range in size from 3 to 7 by 4 to 29 μm. By comparison, *T. loubieri* is characterized by the formation of fusiform giant cells. Although enlarged cells were noted in this isolate that separated it from other *Trichosporon* species, the cells were notably smaller than those seen for *T. loubieri.*

T. mycotoxinivorans is urease positive, grows on cycloheximide medium, and reliably assimilates a variety of carbohydrates, including rhamnose, which helps distinguish it from some of the other *Trichosporon* species and the genus *Geotrichum* (10, 11) but not from *T. loubieri.* Growth characteristics which differentiate the species from *T. loubieri*, as originally described by Molnar et al., are the inability to grow at or above 40°C and assimilation of inulin and galactitol (20). In reviewing our isolates, however, the growth temperature maximums appeared to be variable, as four isolates grew well at 42°C, including the case isolate, two demonstrated weak growth at this temperature, and one was negative for growth at this temperature (Table 2). Inulin and galactitol are not included in the API 20C systems, and so tests for the assimilation of inulin and galactitol were not performed.

This case highlights several potential pitfalls for clinicians and laboratory personnel confronting a similar case. First, correct identification of the organism is complicated by inconsistent results on standard biochemical testing and the various morphologies of species within the genus (26). Indeed, the extent to which the organism might be evaluated can be called into question, particularly if light growth is identified, given that *Candida* species are commonly cultured from the sputum of cystic fibrosis patients, particularly those receiving inhaled aminoglycosides, but are generally not thought to be pathogenic (4). Molds, particularly *Aspergillus* spp., are commonly recovered from patients with cystic fibrosis, but they are of significance only in specific clinical situations, such as after transplantation or in patients with allergic bronchopulmonary aspergillosis (27). Although the cystic fibrosis patient described here was receiving chronic inhaled steroids supplemented by a short 3-day systemic course, in the absence of a central line or past solid organ transplant, this patient would not typically fall into a category considered at risk of invasive fungal disease. The combination of a low prediagnosis suspicion and the intrinsic delay in establishing a genus-specific diagnosis can re-

sult in a clinically significant delay in both the initiation of pathogen-specific antifungal therapy and the empirical selection of effective agents. The interaction of the effects of corticosteroid treatment, chronically impaired pulmonary function, and malnutrition on the pathogenicity of this organism bears further study. Each of these factors likely played an important role in the patient's demise. Nevertheless, the overwhelming involvement of lung tissue (documented histologically) indicates that the likely primary cause of death was *Trichosporon* pneumonia.

Amphotericin B and lipid formulations as well as the echinocandins have limited efficacy against *Trichosporon* species. Triazoles are considered the therapeutic class of choice on the basis of in vitro data, animal models, and individual case descriptions (1, 2, 8, 9, 15, 30, 31). Among the limited options among the antifungal triazoles, voriconazole may be preferred on the basis of its intravenous and oral formulations, as well as its bioavailability. Fluconazole is another option; however, the maximum concentration in plasma/MIC ratio of voriconazole appears to be higher than that of fluconazole. Itraconazole and posaconazole are other options, but their bioavailabilities in seriously ill patients may be uncertain.

The case described here demonstrates that cystic fibrosis patients, particularly those compromised by poor lung function, a high-risk nutritional-metabolic status, and, possibly, the use of corticosteroids, may be at risk of uncommon invasive fungal diseases, as exemplified by the *T. mycotoxinivorans* disease described here. Vigilance and a high index of suspicion should be maintained when fungal isolates from cultures of sputum from symptomatic cystic fibrosis patients are evaluated. *Trichosporon mycotoxinivorans* is an emerging pathogen with unique growth and morphological characteristics. To confirm its identity, however, DNA analysis may be required. The epidemiological link to cystic fibrosis patients requires further study. Patients suspected of deep infection with *T. mycotoxinivorans*, or any *Trichosporon* species for that matter, should receive empirical therapy with voriconazole pending the results of antifungal susceptibility testing. Further research into the ecological niche and the pathogen-host interactions of *T. mycotoxinivorans* is warranted.

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Fatal *Actinomucor elegans* var. *kuwaitiensis* Infection following Combat Trauma▽

Charla C. Tully,[1] Anna M. Romanelli,[2] Deanna A. Sutton,[3,4] Brian L. Wickes,[2,4] and Duane R. Hospenthal[4,5]*

Department of Medicine, Wilford Hall Medical Center, Lackland Air Force Base, Lackland,[1] Department of Microbiology and Immunology[2] and Fungus Testing Laboratory, Department of Pathology,[3] University of Texas Health Science Center at San Antonio, San Antonio, San Antonio Center for Medical Mycology, San Antonio,[4] and Infectious Disease Service, Department of Medicine, Brooke Army Medical Center, Fort Sam Houston, Houston,[5] Texas

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We report the first case of invasive mucormycosis secondary to *Actinomucor elegans* infection. A severely injured soldier with a fatal *A. elegans* var. *kuwaitiensis* infection is described. The identification of this fungus was performed by classical and molecular methods, and this report documents the pathogenicity of the recently described variety *Actinomucor elegans* var. *kuwaitiensis*.

CASE REPORT

A previously healthy 30-year-old male was injured by an improvised explosive device in Iraq, sustaining extensive wounds to his right side. He was evacuated to a military hospital in Iraq and taken immediately to the operating room for complex pelvic fracture debridement and fixation, right lower extremity disarticulation, right through-the-elbow amputation, and an exploratory laparotomy. He was stabilized and evacuated to a military medical center in Germany. After further evaluation and stabilization, including washing out of his right flank, hip, and forearm and washing out of his abdomen without evidence of bowel injury, a wound vacuum-assisted closure device was placed over his open abdomen, and he was transferred to Brooke Army Medical Center (BAMC) for further care.

The patient was febrile upon arrival to BAMC. A left subclavian central venous catheter that was placed while the patient was in the field was replaced over a wire, and blood, urine, and respiratory cultures were performed. Rare fungal elements noted in the respiratory sample were later identified as an *Aspergillus* species. This fungus was not recovered subsequently, and blood and urine cultures remained negative. Broad-spectrum antibiotics were started for empirical antibacterial therapy, and the patient was taken to surgery for wound exploration and debridement. The pelvic wound was noted to extend into the peritoneal cavity, exposing the right lobe of the liver, right kidney, and small bowel, all of which appeared healthy. Gram stains of wound cultures taken from the right ilium and the iliac vein were unremarkable, and cultures ultimately grew no organisms. On day 4 postinjury, the patient was again febrile, and urine, blood, and sputum samples were sent for Gram staining and culture. Evaluation of the sputum revealed yeast, identified 3 days later as *Arthrographis kalrae*. This fungus was not isolated again, and its identification was not further confirmed. On day 5, the patient's temperature increased to 103°F, and his left subclavian central venous catheter was removed and cultured, revealing no growth. Devitalized tissue in the hip and hamstring and a portion of the rectus muscles on the right were debrided. A wound vacuum-assisted closure device was placed over the right hip wound, which communicated with the abdomen. Given the patient's persistent fever on day 6, blood and urine cultures were sent for testing. The patient's clinical status continued to deteriorate, with worsening hypotension, oliguria, and evidence of rhabdomyolysis. On day 7, the patient was once again taken to surgery for debridement. He was noted to have areas of necrosis involving multiple abdominal and gluteal muscles, with skin necrosis that extended across the midline. Extensive debridement of these areas was performed, and tissue from the right hip was submitted for culture. On day 8, the patient again underwent extensive debridement of necrotic tissue. Muscles surrounding the pelvis and extending into the abdomen were further debrided. Wound necrosis was widespread and too substantial to fully remove. Necrotic tissue extended up the anterior wall, involving the intercostals, the psoas, and paraspinal muscles, and extended throughout the bowel (including the stomach), liver, and right kidney. Sections from bowel and muscle were sent for examination intraoperatively, and touch prep of tissue was remarkable for fungal elements. He was given a dose of caspofungin in the operating room and subsequently started on fluconazole for treatment of candidemia (identified as *Candida tropicalis*) found on blood cultures obtained on day 6. The health care team felt that the patient's wounds and extent of tissue necrosis were beyond survivability. The prognosis was discussed in detail with his family, who opted to transition to comfort measures on the afternoon of day 9. The patient expired later that evening.

Tissue from debridement of the right hip collected on day 7 was suspicious for a mucormycosis by histopathology—later confirmed by culture as an *Actinomucor elegans* infection. Specimens submitted for histopathology on day 8 were later noted to have diffuse invasive fungal infection with frequent lymphovascular invasion. Gomori methenamine silver (GMS) staining revealed fungal structures consistent with a mixed

* Corresponding author. Mailing address: Infectious Disease (MCHE-MDI), Brooke Army Medical Center, 3851 Roger Brooke Drive, Fort Sam Houston, TX 78234. Phone: (210) 916-4355. Fax: (210) 916-0388. E-mail: duane.hospenthal@amedd.army.mil.

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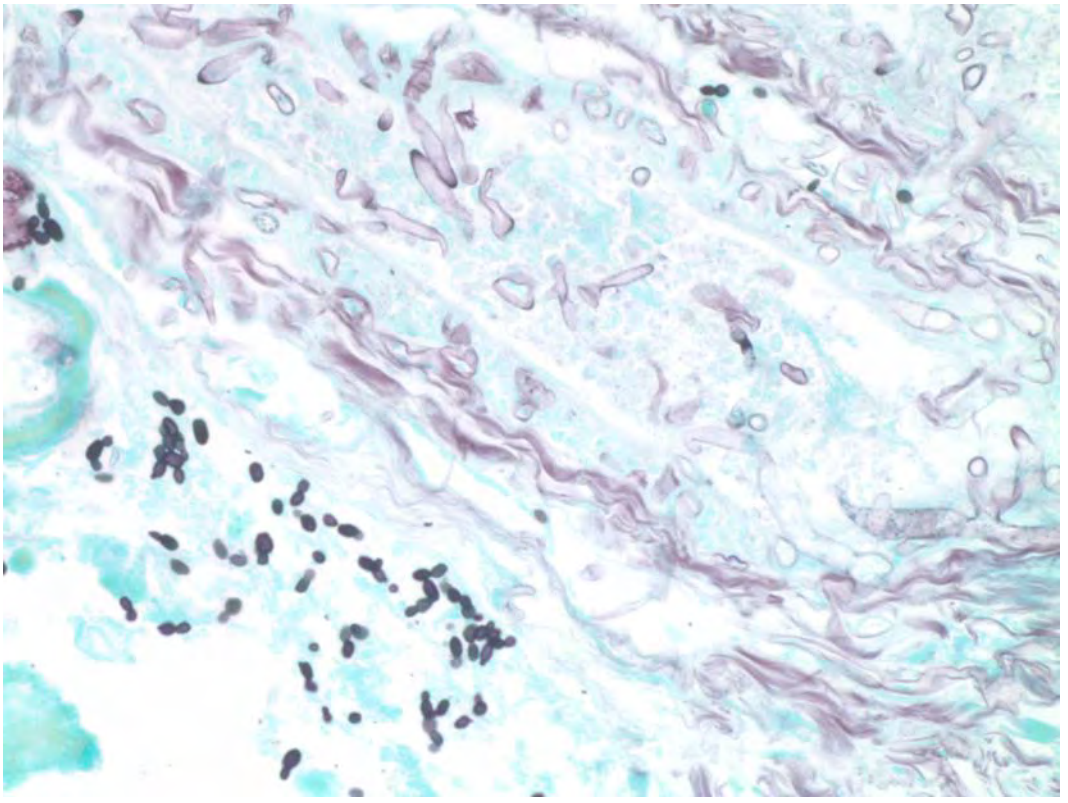

FIG. 1. Gomori methenamine silver stain of necrotic intra-abdominal muscle showing invasive, ribbon-like, branching fungal elements characteristic of members of *Mucorales* species together with budding yeasts characteristic of *Candida* species.

mucormycosis/candidiasis (Fig. 1). At autopsy, postmortem tissue examination showed intravascular and parenchymal fungal elements that were consistent with a dual population of *Candida*- and *Mucor* species-like mold, and cultures ultimately grew *Candida tropicalis* and *Actinomucor elegans*, the latter of which was identified 18 to 21 days postmortem. Involved organs included lungs, stomach, small and large bowels, liver, spleen, pancreas, adrenal glands, kidneys, prostate, and bladder. Pathology determined the cause of death to be a dual-population, disseminated, invasive fungal infection of *Candida* and Mucorales complicating multiple blast injuries.

Phenotypic identification of *Actinomucor*. *Actinomucor elegans* was identified by morphological features and molecular characterization. An isolate from the right leg stump was forwarded to the Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio (UTHSCSA), and accessioned into the stock collection as UTHSC 07-831. There, the sterile, woolly, white isolate was subcultured onto 60-mm Czapek Dox agar plates (prepared in-house) at 25°C and subjected to a water culture at 37°C in an effort to induce sporulation, as the frequently sterile Mucorales *Apophysomyces elegans* and *Saksenaea vasiformis* were under consideration. It was also subcultured on potato flake agar (prepared in-house) tubes at 25, 37, 40, and 45°C for temperature studies. The isolate grew rapidly at 25°C, filling the 60-mm Czapek Dox agar plate in 2 days. Colonies were initially white and woolly, becoming yellowish brown after 4 days of incubation, and displayed a pale-yellow diffusing pigment. The isolate also grew rapidly in the 37°C water culture. Temperature studies revealed growth up to 40°C but not at 45°C. Salient microscopic features included verticillately branched sporangiophores of various lengths (Fig. 2), commonly delicately roughened below the septum (Fig. 3), with secondary branches being borne beneath and to the side of septa below the primary sporangium (Fig. 2 and 4). At maturity, sporangia were covered with prominent spine-like projections on their outer walls, and small, barely visible collarettes were present. Sporangiospores were smooth to very delicately spiny as observed by light microscopy, were nearly globose, and measured 6 to 8 μm in diameter (Fig. 2 to 4). Rhizoids were present. On the basis of these features, the isolate was identified as *Actinomucor elegans* and deposited into the University of Alberta Microfungus Collection and Herbarium as strain UAMH 10999.

Genotypic identification of *Actinomucor*. To determine the etiologic agent, a sequence-based approach using both the internal transcribed spacer (ITS) and D1/D2 ribosomal DNA regions as targets for the molecular identification of 07-831 was performed, followed by a TA cloning step to yield a plasmid that would serve as a sequencing template.

The case isolate was grown for 18 h at 30°C on potato dextrose agar (Difco, Detroit, MI). A small number of hyphae were scraped off each plate and suspended in 50 μl of Prepman Ultra reagent (Applied Biosystems, Foster, CA) in a 0.5-ml microcentrifuge tube. The suspension was vortexed initially for 45 s to 1 min and then heated for 15 min at 100°C. The suspension was then pelleted for 5 min at maximum speed in a

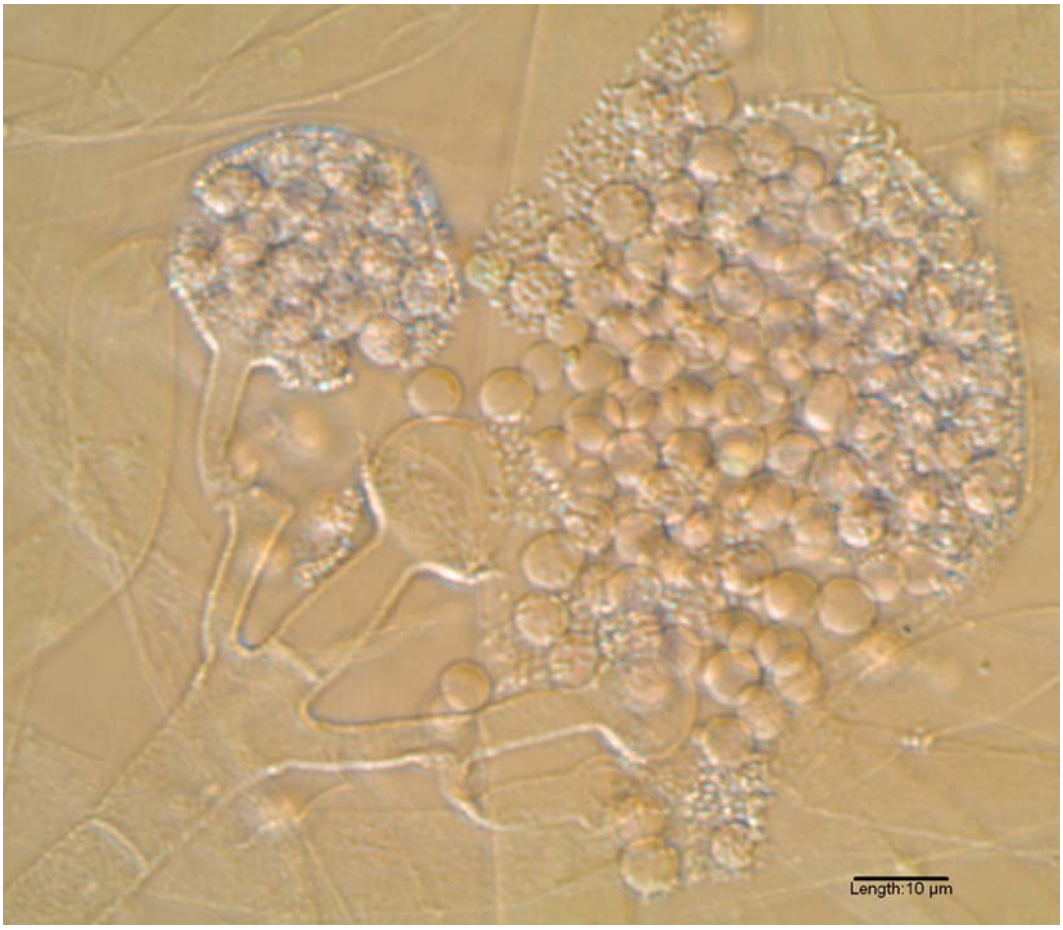

FIG. 2. Verticillately branching sporangiophores, spiny, encrusted sporangia, and sporangiospores of *Actinomucor elegans* var. *kuwaitiensis*. Note secondary sporangiophores being formed beneath the septum on the primary sporangium.

microcentrifuge according to the manufacturer's instructions. The supernatant was transferred to a new tube and stored on ice until PCRs could be set up.

PCRs were performed directly on 3 μl of the Prepman supernatant in a 50-μl reaction mixture using high-fidelity PfX50 DNA polymerase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. ITS amplicons were obtained using primers (ITS1 and ITS4) and PCR conditions as previously described (20). D1/D2 PCR amplicons were obtained using primers (NL-1 and NL-4) and PCR conditions as described previously (14, 16). Amplifications were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA) using the preprogrammed three-step protocol as the standard program for all reactions and consisted of 35 cycles using an annealing temperature of 59°C and a 1-min extension time. Since the PCR product was going to be used in TA cloning, an additional A-tailing procedure was performed due to the blunt-ended fragments generated by PfX50 amplification. Tailing was performed at the end of the PfX50 cycling program by adding 1 μl of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) to the PfX50 reaction mixture and incubating at 72°C for 30 min. A 5-μl aliquot of the PCR mixture was run on a 0.7% agarose gel and stained with ethidium bromide to confirm amplification, and then the remaining PCR mixture (45 μl) was purified using the Wizard SV gel and PCR cleanup system (Promega, Madison, WI). Three microliters of the purified PCR product was used for TA cloning into pGEM-T Easy (Promega, Madison, WI), which was performed according to the manufacturer's instructions. TA-cloned plasmid DNA was obtained using a Qiaprep spin miniprep kit (Qiagen, Valencia, CA) and then sequenced at the UTHSCSA advanced nucleic acids core facility using vector-derived primers (T7 and SP6) in order to identify the insert sequence. Sequences were then used to perform individual nucleotide-nucleotide searches of the ITS and D1/D2 regions using the BLASTn algorithm at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Identifications were calculated based on percentages of identity from the top three BLAST searches for both the ITS and D1/D2 regions.

BLASTn results for the 07-831 ITS region returned the three highest percentages of identity with (i) *Actinomucor elegans* var. *kuwaitiensis* accession no. AJ849551.1, 99.4% (713 of 717 bp); (ii) *Actinomucor elegans* accession no. FJ176396.1, 99.0% (709 of 716 bp); and (iii) *Actinomucor elegans* accession no. AM745429.1, 99.0% (709 of 716 bp). The three highest percentages of identity for the D1/D2 region were for (i) *Actinomucor elegans* var. *kuwaitiensis* accession no. AJ849552.1, 100% (726 of 726 bp); (ii) *Actino-*

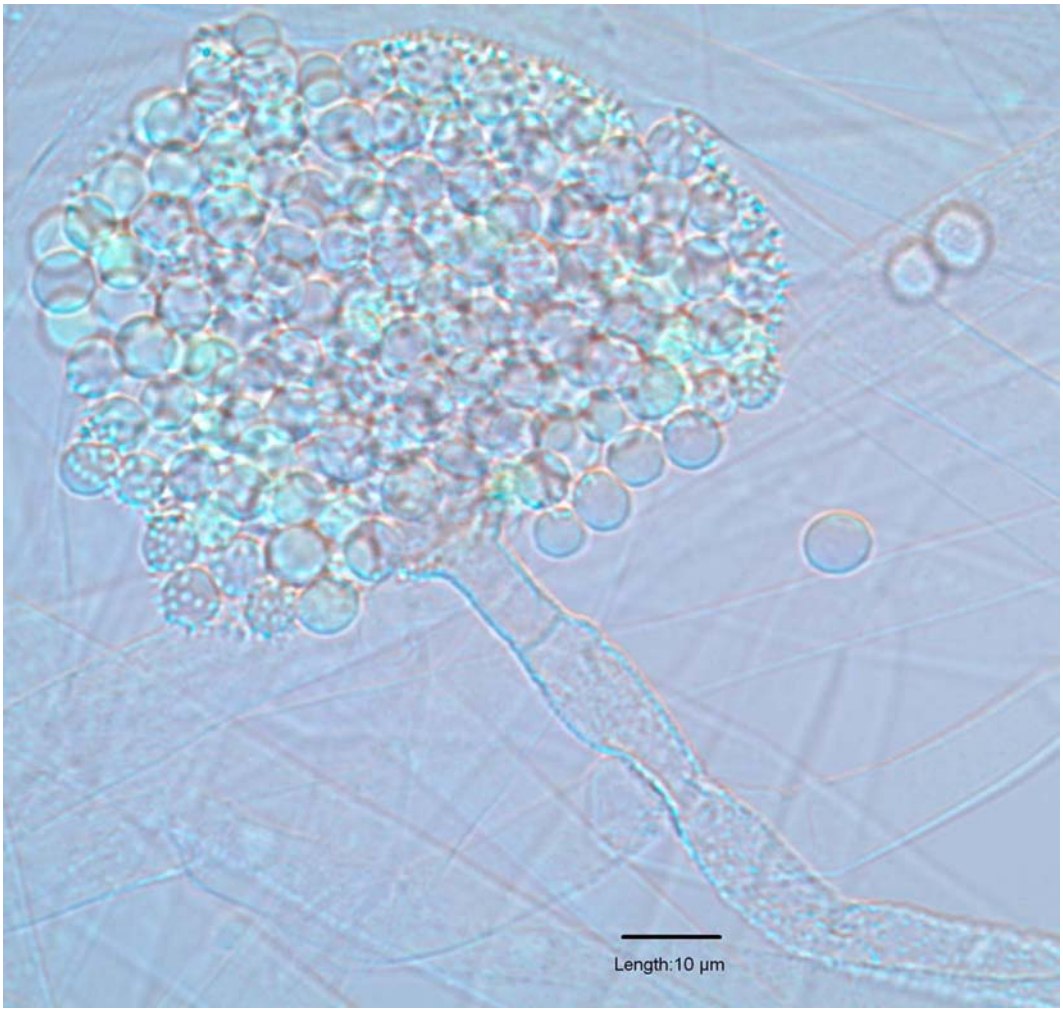

FIG. 3. Single sporangium and sporangiospores of *Actinomucor elegans* var. *kuwaitiensis*. Note the roughened area on the sporangiophore beneath the septum.

mucor elegans var. *meitauzae* accession no. AM745436.1, 99.2% (720 of 726 bp); and (iii) *Actinomucor elegans* accession no. AM745435.1, 99.2% (720 of 726 bp). Although the top three hits for 07-831 had less than 1% sequence divergence within the ITS and D1/D2 regions, among previously described *Actinomucor* varieties, a variety level identification matching *A. elegans* var. *kuwaitensis* was obtained for UTHSC 07-831.

Discussion. The Mucorales are an order of fungi ubiquitous in soil and known to sometimes cause life-threatening infections, termed mucormycosis (zygomycosis), in immunocompromised patients. Mucormycosis has also been reported to occur in immunocompetent victims following trauma (1, 3, 4, 6, 7, 9, 12, 17, 19). Previously reported genera known to cause invasive mucormycoses include *Absidia*, *Rhizopus*, *Mucor*, and *Rhizomucor*, as well as *Apophysomyces*, *Saksenaea*, *Cunninghamella*, *Syncephalastrum*, and *Cokeromyces* (18). Mucormycosis carries a high risk of morbidity and mortality due to the propensity of the fungi to produce vascular invasion and spread hematogenously, leading to dissemination and tissue necrosis (1). As demonstrated with our patient, a high degree of suspicion for invasive fungal infections is needed for early recognition and proper antifungal therapy in order to decrease morbidity and mortality.

Mucormycosis is generally acquired through inhalation of sporangiospores, leading to rhinocerebral and pulmonary disease in immunocompromised hosts (8). In trauma and burn victims, mucormycosis usually starts with direct inoculation of an open wound with contaminated soil rich in organic material (8) or colonization of open wounds via contaminated dressings or during surgical procedures in a hospital setting (18). Trauma and burn patients in intensive care units commonly have multiple associated comorbidities (including organ dysfunction, contaminated wounds, bacterial sepsis, and prolonged courses of broad-spectrum antibiotics), many of which also contribute to compromised immune function, placing the patient at higher risk for developing mucormycosis (3). The levels of virulence of the Mucorales appear to vary, with diseases ranging from contamination of open wounds to production of severe systemic infection (3). Cutaneous mucormycosis is the most common presentation in trauma patients, often

FIG. 4. Verticillately branching sporangiophores, bare columella dislodged from the remainder of the sporangium, and variously sized smooth sporangiospores of *Actinomucor elegans* var. *kuwaitiensis*.

characterized by indurated, dusky-appearing nodules surrounded by a pale halo (3). In a comprehensive review of 929 patients with zygomycosis (mucormycosis), penetrating trauma accounted for 34% of those presenting with cutaneous involvement. Of those with cutaneous involvement, 24% had extension into surrounding muscle or bone and 20% experienced hematogenous dissemination from the skin to other organs (17). Invasive infection associated with vascular invasion may result in thrombosis and infarction of surrounding tissues (3). The type and location of injury are significant in predicting the morbidity and mortality of trauma patients. Of trauma and burn patients infected with *Saksenaea*, two-thirds of patients with abdominal wounds and 100% of those with sinusitis died despite surgery (19). Another study of mucormycosis in trauma patients found a 50% mortality rate among patients with head or trunk involvement, and two-thirds of patients with limb involvement required amputation (3). Aggressive treatment of mucormycosis is required in cases of trauma and burns, with removal of contaminated and necrotic tissue and concurrent treatment with antifungal agents, typically intravenous amphotericin B (3, 4, 9, 19).

Actinomucor (order Mucorales) is a facultative anaerobe ubiquitous in nature (13) and has not previously been identified as a causative agent of invasive mucormycosis (zygomycosis). The genus *Actinomucor* was first described by Schostakowitsch in 1898 (17a) and then again by Benjamin and Hesseline in 1957 (2, 11). *Actinomucor* was noted to be similar to *Mucor*, *Rhizopus*, and *Absidia* species, with broad, irregular sparsely septate hyphae (15). *Actinomucor* is differentiated from *Mucor* by having branched stolons giving rise to rhizoids and sporangiophores and from *Rhizopus* and *Absidia* by the limited growth of stolons and by the arrangement of the columellae and sporangiophores. The genus *Actinomucor* was previously thought to contain two species, *A. elegans* (2) and *A. taiwanensis* (10). *Actinomucor elegans* was differentiated from *A. taiwanensis* by the smaller size of the sporangiospores, 5 to 8 μm versus 7 to 15 μm, and by a maximum growth temperature of 32°C versus 37°C. A study by Zheng and Liu in 2005 (21), however, characterizing 110 strains of *Actinomucor* by ITS region sequencing, demonstrated that the genera *Mucor* and *Actinomucor* are more closely related to one another than to the genus *Rhizopus* and that *Mucor meitauzae* (synonymous with *Actinomucor taiwanensis*) is actually a variety of *A. elegans.* Zheng and Liu, therefore, reduced *A. taiwanensis* to a varietal status of *A. elegans* now known as *A. elegans* var. *meitauzae.* Thus, the genus contained one species with two varieties, *A. elegans* var. *elegans* and *A. elegans* var. *meitauzae. Actinomucor* species are known for their association with the production of soy-based products, providing flavor and texture to food (11).

Actinomucor elegans has been shown to be possibly pathogenic for humans in only two cases reported in the literature. The first case, reported in 2001, was that of an immunocompetent 11-year-old girl diagnosed with maxillary sinusitis. *Actinomucor elegans* was recovered in a culture of maxillary sinus contents; however, invasive disease was not confirmed by histopathology, nor was eosinophilia reported (5). In 2008, an *Actinomucor* species was isolated from a swab of a foot ulcer of a patient with diabetes. Once again, invasive disease was not confirmed by histopathology; however, sequencing of the ITS and D1/D2 regions of this isolate determined that it was a new

variety of *A. elegans*, *A. elegans* var. *kuwaitiensis* (Kw823), with the varietal name being based upon the geographic region of recovery. Although the D1/D2 region of Kw823 demonstrated nucleotide differences from *A. elegans* isolate ATCC 22814 of >1%, it also exhibited a nucleotide difference of <1% from the same region of other strains of *A. elegans* var. *elegans* and *A. elegans* var. *meitauzae*. As this isolate also did not demonstrate unequivocal phenotypic features, sequence data combined with morphology did not seem to support a separate species status. The isolate was also inoculated into immunocompetent mice to prove its in vivo pathogenicity, resulting in 100% mortality in the mouse model (11). Additional phenotypic studies determined that temperature maximums were not useful to separate varieties, as the reference strains *A. elegans* var. *elegans* and *A. elegans* var. *meitauzae* as well as the new variety demonstrated growth at up to 40°C.

Our patient was probably inoculated with *Actinomucor elegans* var. *kuwaitiensis* through his open skin at the time of injury. The concurrent *Candida tropicalis* infection was more likely nosocomial, and the other isolated fungi—*Arthrographis* and *Aspergillus* spp.—were most likely contaminants, as they were not further isolated. The patient's comorbidities—which included acute kidney injury secondary to rhabdomyolysis, multiple fractures requiring surgical intervention, and prolonged use of broad-spectrum antibiotics—likely contributed to a degree of immune suppression, providing an adequate environment for the fungi to grow. As with other cases of mucormycosis in trauma or burn victims, diagnosis of our patient's *Actinomucor* infection was not made in time to allow early initiation of antifungal therapy. It is clear that the medical team adequate cultures and remained abreast of the results. Unfortunately, mucormycosis was not in the differential, nor did the cultures suggest this fatal infection until too late. Mucormycosis should be considered a cause of rapidly progressive necrotic wounds in patients with the previously mentioned risk factors, as it may prove to be fatal unless treated early and adequately with surgical debridement and intravenous antifungal therapy.

Nucleotide sequence accession numbers. Sequences for *Actinomucor elegans* UTHSC 07-831 were deposited in GenBank under accession numbers FJ896015 (ITS) and FJ896016 (D1/D2).

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Cerebral Aspergillosis Caused by *Aspergillus granulosus*▽

Deanna A. Sutton,[1]* Brian L. Wickes,[2] Anna M. Romanelli,[2] Michael G. Rinaldi,[1] Elizabeth H. Thompson,[1] Annette W. Fothergill,[1] Megan K. Dishop,[3,4] Okan Elidemir,[3,4] George B. Mallory,[3,4] Siby P. Moonnamakal,[3,4] Adekunle M. Adesina,[3,4] and Marc G. Schecter[3,4]

Department of Pathology[1] and Department of Microbiology and Immunology,[2] University of Texas Health Science Center at San Antonio, San Antonio, Texas, and Baylor College of Medicine[3] and Texas Children's Hospital,[4] Houston, Texas

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Disseminated disease by *Aspergillus granulosus* has been reported only once previously in a cardiac transplant recipient. We report a fatal central nervous system infection in a lung transplant recipient. Key features of this species in the section *Usti* include growth at 37°C and large, randomly spaced aggregates of variably shaped Hülle cells.

CASE REPORT

An 18-year-old male with bronchiolitis obliterans following a bone marrow transplant and graft-versus-host disease underwent en-bloc double-lung transplantation with a tracheal anastomosis with bronchial artery revascularization in October 2006. Ten years previously he had been diagnosed with pre-B-cell acute lymphocytic leukemia. He had undergone three courses of chemotherapy with three subsequent relapses. One year prior to transplant, he underwent allogeneic bone marrow transplantation. Post-bone marrow transplant, the patient developed cytomegalovirus infection, *Aspergillus terreus* pneumonia, *Mycobacterium avium* complex (MAC) infection, and influenza A. He received antifungal treatment with voriconazole, amphotericin B lipid complex (ABLC), caspofungin, and posaconazole. MAC was treated with ethambutol, azithromycin, and levofloxacin. At the time of lung transplant, he had no known active infections.

Donor lung culture revealed methicillin-resistant *Staphylococcus aureus*, beta *Streptococcus* group F, and alpha *Streptococcus*. He was extubated, weaned from oxygen, and discharged on day 9 posttransplant (PT). He was readmitted on day 10 PT for seizures. A computed tomography scan of the brain revealed a subtle hypodensity in the left frontal lobe and the middle frontal gyrus, which was thought possibly to be an artifact versus an old ischemia or contusion. The seizure was attributed to tacrolimus toxicity, as his tacrolimus trough blood level was elevated at 34.8 ng/ml. During this admission, he developed respiratory distress and was intubated. Bronchoscopy on day 13 PT revealed mucosal sloughing and accumulated mucus, debris, and old blood. Bronchoalveolar lavage was sent for bacterial, viral, and fungal cultures with isolation of a *Bipolaris* species, which was not considered significant. After debridement, the patient was breathing easily, extubated, and discharged home the same day.

Following discharge, he developed headaches and subsequent seizures without loss of consciousness despite seizure prophylaxis. A fundoscopic exam on day 14 PT showed no signs of increased intracranial pressure. He underwent elective flexible bronchoscopy on day 16 PT, and bacterial, mycobacterium, viral, and fungal cultures from bronchoalveolar lavage fluid were unremarkable (10,000 oropharyngeal flora). On day 17 PT, he developed a low-grade fever and altered mental status. A lumbar puncture revealed 1,110 white blood cells/mm^3 with 79% neutrophils, a cerebrospinal fluid (CSF) protein level of 109 mg/dl, and a CSF glucose level of 41 mg/dl, with a serum glucose level of 122 mg/dl. He was hospitalized and started on empirical intravenous vancomycin, cefotaxime, metronidazole, ABLC, and oral voriconazole. The initial CSF culture, sent for bacterial culture, India ink stain, and cryptococcal antigen, grew *Staphylococcus warnari*. Magnetic resonance imaging scan of his brain showed multifocal ring-enhancing lesions at the gray-white junction of both cerebral hemispheres and early ventriculitis. The initial magnetic resonance angiography showed only a slight irregularity of the vessels near some of the lesions, and the magnetic resonance venography was normal. The size and the location of the lesions did not lend themselves to an accessible brain biopsy. A computed tomography scan of the chest showed two small nodules in the left lower lobe, which were in the region of previous transbronchial biopsy sites. Septic emboli of infectious origin were suspected.

On day 55 PT, he started experiencing transient ischemic attacks, which manifested as acute aphasia and right-sided hemiparesis. Over the following month, he gradually became more neurologically impaired. As his disease progressed, posaconazole was added. Serial magnetic resonance imagings and magnetic resonance angiographies showed progression of vascular occlusion, particularly in the left internal carotid artery and distally in the left middle cerebral and posterior cerebral arteries but no significant change in the size and number of the ring-enhancing lesions. Cerebral arteriograms confirmed occlusion of the left internal carotid artery in addition to a sagittal sinus thrombus and evidence of collateral formation.

* Corresponding author. Mailing address: Fungus Testing Laboratory, Department of Pathology—MC 7750, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900. Phone: (210) 567-4131. Fax: (210) 567-4076. E-mail: suttond@uthscsa.edu.

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As his vascular disease progressed, he developed ophthalmoplegia, significant tremor, aphasia, and worsening comprehension and communication capabilities. The occlusion of his cerebral vasculature continued to progress despite maximal antimicrobial therapy. He developed multiple areas of cerebral infarction, including the left medial temporal lobe, left caudate, and left internal capsule. An empirical course of ethambutol, clarithromycin, and levofloxacin was started day 76 PT for treatment of MAC. He had temporary halting of disease progression with courses of intravenous immunoglobulin for hypogammaglobulimemia, but this effect dissipated with subsequent doses. Antibiotics for the empirical treatment of MAC were stopped on day 83 PT. ABLC and posaconazole were continued until he was made "do not resuscitate" day 85 PT. The patient was transferred to a hospice facility in the community and died peacefully 2 days after hospital discharge on day 87 PT.

Autopsy examination of the brain showed an organizing exudate over the base of the brain and brain stem, with occlusive thrombosis of the basilar artery and left middle cerebral artery. There was left hemispheric infarction involving the frontotemporal and parietal lobes, left basal ganglia, and left thalamus, as well as severe cerebral edema with bilateral uncal herniation. The arterial thromboses were caused by an angioinvasive fungus, which extended through the arterial wall and elicited a necrotizing granulomatous reaction in the surrounding meninges (Fig. 1A and B). The nonpigmented fungal hyphae branched at acute angles and showed frequent septations, typical of *Aspergillus* and/or several other species (Fig. 1A, inset). Autopsy of the lungs revealed *Enterobacter cloacae* but no evidence of rejection or the presence of any fungal organisms, including *Aspergillus terreus*.

Morphological and molecular characterization. An isolate recovered from the autopsy brain stem was forwarded to the Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center at San Antonio, and accessioned into their stock collection as UTHSC 07-462. An initial tease of the isolate, tan and granular, revealed many globose to elongate Hülle cells but no fruiting heads of *Aspergillus*. The isolate was subcultured onto potato flakes agar (PFA), carnation leaf agar, and Czapek Dox agar (CZA), prepared in-house, and incubated at 25°C for demonstration of macroscopic and microscopic features. Slide culture preparations and temperature studies (25, 35, 40, and 45°C) were conducted on PFA. At 2 weeks (PFA at 25°C), colonies were cream to white and floccose at the periphery and buff to yellowish tan centrally, with a very slight clear exudate (Fig. 2A, top). Colonies on CZA were mostly cream, more irregularly furrowed, and exhibited reduced conidiation (Fig. 2A, bottom). The central granular areas on PFA consisted of colorless masses of thick-walled (6 to 8 μm), predominately globose but also oval to elongate to irregularly shaped Hülle cells with individual, mature cells ranging from 20 to 40 μm. The slide culture was also significant for profuse clusters of Hülle cells (Fig. 2B). Robust *Aspergillus* fruiting heads were sparse and were borne on long (200 to 480 μm), subhyaline to brown, smooth, thick-walled conidiophores terminating in small (12 to 18 μm wide by 15 to 25 μm long), oval to elliptical vesicles. Metulae and bottle-shaped phialides of almost equal length (3.5 to 5.5 μm) covered most of the surface of the vesicle (Fig. 2C). Conidia were pale green in mass, globose, and finely echinulate, measuring 3.5 to 5.5 μm in diameter (Fig. 2C and D). More commonly, fruiting structures were reduced in size with small, *Penicillium*-like vesicles or were single chains of conidia borne from solitary phialides (Fig. 2D). The isolate grew well at 25, 35, and 40°C but failed to grow at 45°C. On the basis of these features, it was tentatively identified as *Aspergillus granulosus* (2, 3, 8, 15, 21) and was deposited into the University of Alberta Mold Herbarium under accession no. UAMH 10935. The isolate was then further characterized by molecular sequencing under accession no. R-3921.

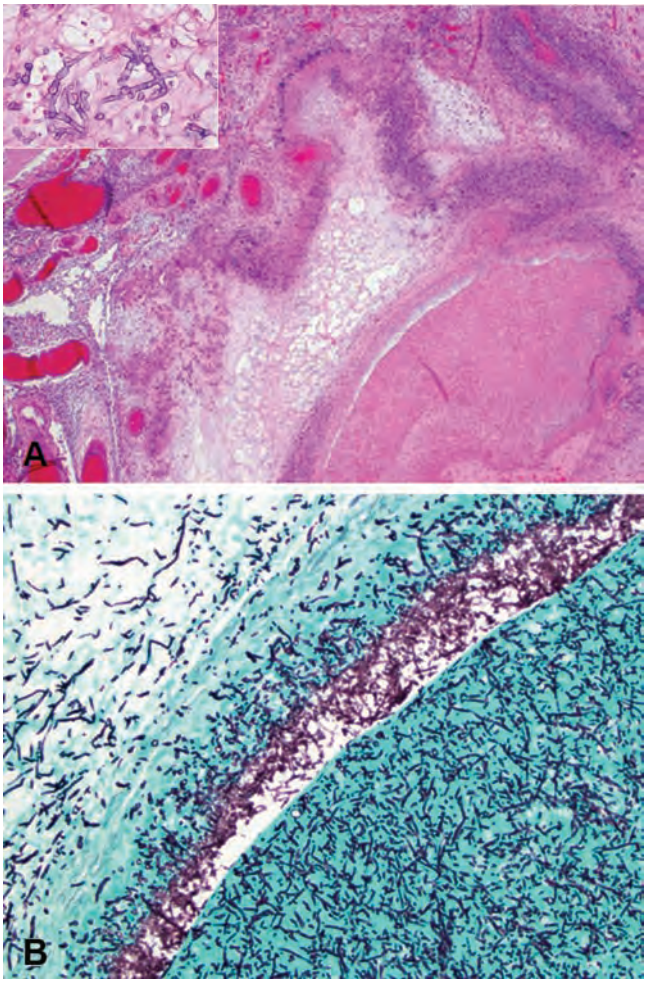

FIG. 1. (A) The thrombosed left middle cerebral artery was surrounded by granulomatous inflammation in the meninges (hematoxylin and eosin; original magnification, ×20). (Inset) The fungal hyphae were nonpigmented and septate (hematoxylin and eosin; original magnification, ×400). (B) Silver stain highlights the fungal hyphae within the thrombus and extending through the arterial wall (Gomori methenamine silver; 100× original magnification).

DNA was prepared from the subcultures of R-3921 and the control type cultures, *Aspergillus granulosus* (NRRL 1932) and *Aspergillus ustus* (NRRL 275). These isolates were obtained from Clete Kurtzman, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL. DNA isolated for each sample was prepared for PCR as previously described by Grant et al. (7).

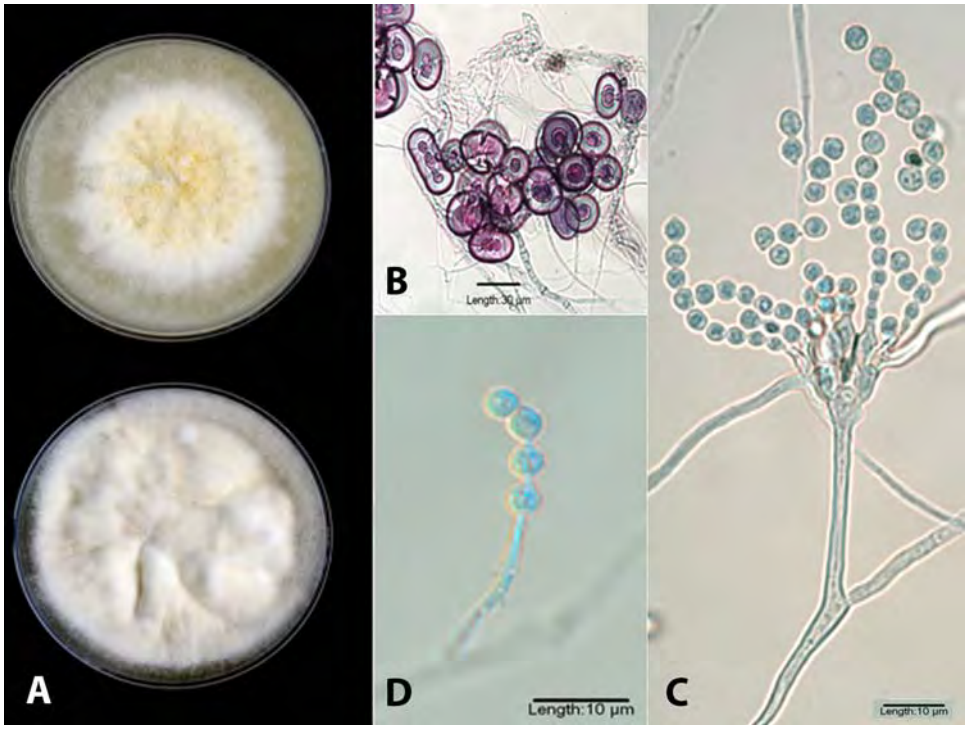

FIG. 2. (A) PFA (top) and CZA (bottom) plates incubated for 14 days at 25°C. (B) Clusters of Hülle cells on the slide culture in lacto-fuchsin mounting medium. (C) Slide culture mounted in lactophenol cotton blue showing a more robust, biseriate fruiting head demonstrating metulae and bottle-shaped phialides of almost equal length. (D) Globose, finely echinulate conidia borne from a solitary phialide (lactophenol cotton blue mount).

DNA amplification by PCR was performed in 50-μl total volume reactions using 3 μl of the Prepman supernatant as template. Each reaction was performed using high-fidelity *Pfx 50* DNA polymerase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Internal transcribed spacer (ITS) and D1/D2 amplicons were obtained using primers ITS1 and ITS4 and NL1 and NL4, respectively (11, 16, 24). PCR amplification was performed in a PTC-100 thermocycler (MJ Research, Watertown, MA), and a standard three-step protocol was used for all reactions, including 30 cycles for each reaction with an annealing temperature of 60°C and a 1-min extension time. To confirm PCR amplification, a 15-μl aliquot of each PCR was run on a 0.7% agarose gel and stained with ethidium bromide. The remaining PCR template was purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA) and then sequenced at the UTHSCSA Advanced Nucleic Acids Core Facility. Each purified template was sequenced on both strands, and the sequences obtained were then used to perform nucleotide-nucleotide searches utilizing the BLASTn database at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/).

BLAST outputs were sorted based on maximum identity, and identifications were made when BLAST searches yielded ≥98% identity for sequences that displayed at least 90% query coverage. β-Tubulin amplicons were obtained using primers bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) under the conditions described by Glass and Donaldson (6) and with a PTC-100 thermocycler (MJ Research, Watertown, MA). PCR products were visualized, cleaned, and sequenced as described above using the PCR primers as sequencing primers. Sequences were then used to perform individual nucleotide-nucleotide searches using the BLASTn algorithm at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Following the classification scheme of Houbraken et al. (8) for *Aspergillus*, the section *Usti* includes eight species: *A. ustus*, *A. puniceus*, *A. granulosus*, *A. pseudodeflectus*, *A. calidoustus*, *A. insuetus*, and *A. keveii*. *A. granulosus* and *A. ustus*, in particular, are morphologically very closely related. *A. ustus* is a variable species, due to the fact that isolates may vary in their colony color from mud brown to slate gray, with colony reverse colors from uncolored through yellow to dark brown (8). Therefore, we examined NRRL 275, NRRL 1932, and R-3921 using sequence analysis of the ITS region and the 28S rRNA gene (D1/D2) of the rRNA gene cluster. A summary of results obtained from the BLASTn searches for the three *Aspergillus* isolates is shown in Table 1.

The molecular identification on the top three BLASTn identities resulted in R-3921 being classified as *A. granulosus*. When the BLAST hits were reviewed with a 98% or higher identity as the cutoff point, *A. ustus* was very close, with 99% identity in the D1/D2 region, demonstrating the close relationship of *A. ustus* to *A. granulosus* at the sequence level for the D1/D2 locus. For the ITS region, however, no *A. ustus* sequence showed a percent identity greater than 97%. Nonetheless, in order to confirm the molecular data we obtained using the ITS and D1/D2 regions, the β-tubulin gene was also examined for further identity confirmation of the *Aspergillus* species. The

TABLE 1. Summary of BLASTn results for the ITS, D1/D2, and β-tubulin regions

Subculture ID	BLASTn ID for ITS region	GenBank accession no. of top 3 ITS hits	% Identity	BLASTn ID for D1/D2 region	GenBank accession no. of top 3 D1/D2 hits	% Identity	BLASTn ID for β-tubulin gene	GenBank accession no. of top 3 β-tubulin hits	% Identity	Identity based on sequence analysis of all 3 regions
A. ustus reference isolate (NRRL 275)	*A. ustus*	EF652492.1	100	*A. puniceus*	EF652498.1	100	*A. ustus*	EF591727.1	100	*A. ustus*
	A. ustus	EF652455.1	100	*A. puniceus*	EF652469.1	100	*A. ustus*	EF591722.1	99	
	A. ustus	AY373877.1	100	*A. ustus*	EF652455.1	100	*A. ustus*	EF591726.1	99	
A. granulosus reference isolate (NRRL 1932)	*A. granulosus*	EF652430.1	100	*A. granulosus*	EF652430.1	100	*A. granulosus*	EF591734.1	99	*A. granulosus*
	A. granulosus	EF652429.1	100	*A. granulosus*	EF652429.1	100	*A. granulosus*	EF591733.1	99	
	A. granulosus	EF591737.1	100	*A. pseudodeflectus*	EF652507.1	99	*A. granulosus*	EF591735.1	98	
R-3921	*A. granulosus*	EF652430.1	99	*A. granulosus*	EF652430.1	100	*A. granulosus*	EF591734.1	99	*A. granulosus*
	A. granulosus	EF652429.1	99	*A. granulosus*	EF652429.1	100	*A. granulosus*	EF591735.1	98	
	A. granulosus	EF591737.1	99	*A. granulosus*	AF433050.1	100	*A. granulosus*	EF591733.1	98	

closest *A. ustus* sequence for R-3921 displayed 87% identity by β-tubulin gene sequencing. Based on the sequence identities using ITS, D1/D2, and β-tubulin regions, R-3921 was identified as *A. granulosus*. Comparison of the ITS and D1/D2 regions along with the β-tubulin gene proved to be satisfactorily discriminatory for the accurate identification of *A. granulosus* and *A. ustus* species.

The type strain of *Aspergillus granulosus* Raper and Thom 1944, isolated in 1942 from soil in Fayetteville, AR, was originally classified in the *A. ustus* group. The assignment of *Aspergillus* species to groups was an early method used to categorize like strains with similar morphological features (19, 20). In 1965, Raper and Fennell (17) transferred the species to the *A. versicolor* group, which was expanded to accommodate species that were thought to be related but were somewhat dissimilar. They did, however, acknowledge that except for the difference in Hülle cells, *A. granulosus* bore a close resemblance to *A. ustus*. In 1985 (4), groups were assigned a taxonomic hierarchy as sections with the placement of *A. granulosus* in the now obsolete section *Versicolores* (15). In 1989, species were further categorized on the basis of their conidial ornamentation as seen with scanning electron microscopy (10), and *A. granulosus* was described as having a lobate-reticulate pattern. More recently, a polyphasic approach has been used to clarify the taxonomic placement of *Aspergillus* species. This has included molecular characterization by multilocus sequence analysis (primarily the ITS region and parts of the β-tubulin, calmodulin, and actin genes) combined with analysis of extrolite profiles and morphological features (8, 15). Although the lineage numbers for section *Usti* differ somewhat between Houbraken et al. (8) and Peterson (15), these analyses have resulted in the unequivocal placement of *A. granulosus* in the *Aspergillus* section *Usti*. It is of interest to speculate that this organism, first described from soil in Arkansas and subsequently recovered in other temperate areas (Texas, Arizona, Costa Rica, and Liberia) (17), may be more prevalent in warmer climates, as the isolates recovered thus far in transplant recipients appear to have been from residents of these areas. The first case reported in New York was at a Veterans' Affairs Medical Center, and the authors do not speculate about where the infection may have been acquired. The ability of the organism to grow at 40°C also suggests its neurotropic potential, as was borne out in this case. *Aspergillus granulosus* is distinguished from *A. calidoustus*, the most common clinically significant species in the section *Usti* and formerly called *A. ustus* (21), by its buff to pale brownish rather than grayish yellow to grayish brown colony, by conidia that are only slight roughened (finely echinulate or verruculose) rather than coarsely roughened to echinulate, by sparse conidial production in some isolates, by a negative Ehrlich reaction (21), and by the preponderance of colorless aggregates of globose to elongate Hülle cells at maturity, giving a granular appearance to the colony. This species also differs significantly in its in vitro antifungal susceptibility pattern, appearing considerably more susceptible than *A. calidoustus*, which demonstrates resistance to several classes of antifungal agents (5, 9, 12–14, 18, 22, 23, 25). In vitro antifungal susceptibility testing of the case isolate according to the previously published Clinical and Laboratory Standards Institute M38-A document for filamentous fungi (1) demonstrated the following MICs: amphotericin B, 0.5 μg/ml; caspofungin (reported as the minimum effective concentration), 0.125 μg/ml; voriconazole, 4 μg/ml; and posaconazole, 1 μg/ml. Two additional isolates from lung transplant recipients (Texas and California) received in the Fungus Testing Laboratory for identification and antifungal susceptibility testing were also confirmed as *A. granulosus*. While these patients are neither part of this case report nor are any clinical details available, Table 2 provides the in vitro susceptibility data for them as well as for the first reported isolate in a cardiac transplant recipient (3). Although no defined breakpoints exist for this organism, in vitro data for most antifungal agents tested for these three isolates would suggest clinical efficacy based upon achievable serum drug concentrations using standard dosing regimens.

A. granulosus may be an underreported species in the section *Usti*, particularly in lung transplant recipients, as it may be difficult to identify morphologically due to poor conidiation. Profuse Hülle cell production, however, would suggest a species in the genus *Aspergillus* or *Emericella*. The ability of some isolates to grow at 40°C, as evidenced by this case, also highlights its neurotropic potential.

Nucleotide sequence accession numbers. GenBank accession numbers are as follows: FJ771045 for the 18S rRNA gene (partial sequence), ITS1, 5.8S rRNA gene, ITS2 (complete

TABLE 2. In vitro antifungal susceptibility data for *A. granulosus*

Isolate (reference)	Antifungal susceptibility (μg/ml) to:				
	Amphotericin B (MIC)	Caspofungin (MEC)[a]	Itraconazole (MIC)	Voriconazole (MIC)	Posaconazole (MIC)
Case isolate	0.5	0.125	NT[b]	4	1
Texas lung transplant	1	0.25	0.5	2	NT
California lung transplant	NT	0.5	NT	0.25	0.5
Cardiac transplant (3)	0.29	NT	1.25	NT	NT

[a] MEC, minimum effective concentration.
[b] NT, not tested.

sequence), and 28S rRNA gene (partial sequence); FJ771046 for the 28S rRNA gene (partial sequence); and FJ771047 for the β-tubulin gene (partial sequence).

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Beauveria Keratitis and Biopesticides: Case Histories and a Random Amplification of Polymorphic DNA Comparison

Brett Pariseau, MS, MD, Sarah Nehls, MD,† Gregory S. H. Ogawa, MD,‡ Deanna A. Sutton, PhD,§ Brian L. Wickes, PhD,‖ and Anna M. Romanelli, MS,‖*

Purpose: The purposes of this study were to describe 2 contact lens–associated *Beauveria* keratitis cases and to compare the isolates of 3 contact lens–associated *Beauveria* keratitis cases with *Beauveria*-based biopesticides using random amplification of polymorphic DNA (RAPD).

Methods: A 55-year-old diabetic woman from New Mexico and a 31-year-old healthy woman from southern Wisconsin developed soft contact lens–related corneal ulcers unresponsive to topical moxifloxacin and prednisolone acetate drops. Their corneal cultures grew *B. bassiana*. These isolates, an isolate from a third soft contact lens–related *Beauveria* keratitis case, and *Beauveria*-based biopesticides sold in the United States were analyzed using morphological features, DNA sequencing, and RAPD. A PubMed, Cochrane Library, OVID, UpToDate, and Google search using the term "Beauveria" found only 9 reported *Beauveria* keratitis infections.

Results: Patient 1 responded to topical natamycin, ketoconazole, and 200 mg oral ketoconazole twice daily before developing a secondary bacterial infection requiring penetrating keratoplasty. After subsequent cataract surgery, the best-corrected visual acuity was 20/20. Patient 2 was treated with topical natamycin, topical amphotericin, and 200 mg oral voriconazole twice daily for 1 month with residual scarring and a best-corrected visual acuity of 20/25. RAPD showed that all isolates were unrelated.

Conclusions: Although earlier reported *Beauveria* keratitis cases occurred after corneal injury in patients who did not wear contact lenses, 3 recent patients wore soft contact lenses and denied trauma, mirroring a changing trend in microbial keratitis. RAPD analysis showed that the *Beauveria* isolates were unrelated to one another and to *Beauveria*-based biopesticides. In Patient 2, oral voriconazole worked well.

Key Words: *Beauveria*, keratitis, voriconazole, biopesticide

(*Cornea* 2010;29:152–158)

Beauveria is an entomopathogenic fungal genus that grows in soil around the world. The most common species, *B. bassiana*, is the active ingredient in commercially produced biopesticides and infects more than 700 insects.[1] *B. bassiana* was the first microorganism proven to cause disease when the Italian entomologist Agostino Bassi discovered that it killed silkworms in 1835.[2] In 1995, a fungus isolated from a beetle in 1977 became the first of 4 *B. bassiana* strains registered with the Environmental Protection Agency for use as biopesticides.[3,4] To date, there have been only 9 *Beauveria* keratitis reports, 6 listed in PubMed. In this article, we present 2 new *B. bassiana* keratitis cases, compare 3 recent contact lens–related *B. bassiana* keratitis isolates with one another and with strains in *Beauveria*-based biopesticides using random amplification of polymorphic DNA (RAPD), and review the literature.

CASE REPORTS

On January 5, 2006, a 55-year-old insulin-dependent diabetic Caucasian woman was referred to Eye Associates of New Mexico for eye irritation after 2 months of treatment with a variety of drops. She took prednisolone and moxifloxacin drops 3 times daily and wore CooperVision Proclear multifocal soft contact lenses (CooperVision, Fairport, NY) cleansed with Bausch & Lomb ReNu with MoistureLoc (Rochester, NY). Prior prednisolone drop tapering failed because of worsening ocular discomfort.

The left eye was painful, and the visual acuity with glasses was 20/100. A 2.2-mm elevated white plaque adhered to the cornea with linear infiltrates in the anterior stroma radiating outward. There was no epithelial defect, and the clinical impression was that of a fungal keratitis (Fig. 1A). Corneal cultures grew *B. bassiana*.

The patient began 200 mg oral ketoconazole twice daily, ketoconazole and natamycin drops every 2 hours while awake, and ketorolac tromethamine drops as needed up to 4 times daily for pain. The isolate was confirmed by morphologic identification as *B. bassiana* by the Fungus Testing Laboratory at the University of Texas Health Science Center, San Antonio, TX. After 12 weeks of treatment, the eye was comfortable with a visual acuity of 20/30.

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From the *Department of Ophthalmology, Monroe Clinic, Monroe, WI; †Corneal Service, Department of Ophthalmology and Visual Sciences, University of Wisconsin, Madison, WI; ‡Eye Associates of New Mexico, Albuquerque, NM; §The Fundus Testing Laboratory, Department of Pathology; and ‖Department of Microbiology, University of Texas Health Science Center at San Antonio, San Antonio, TX.

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Reprints: Sarah Nehls, MD, Department of Ophthalmology and Visual Sciences, University of Wisconsin, 2870 University Avenue, Suite 206, Madison, WI 53705 (e-mail: nehls@ophth.wisc.edu).

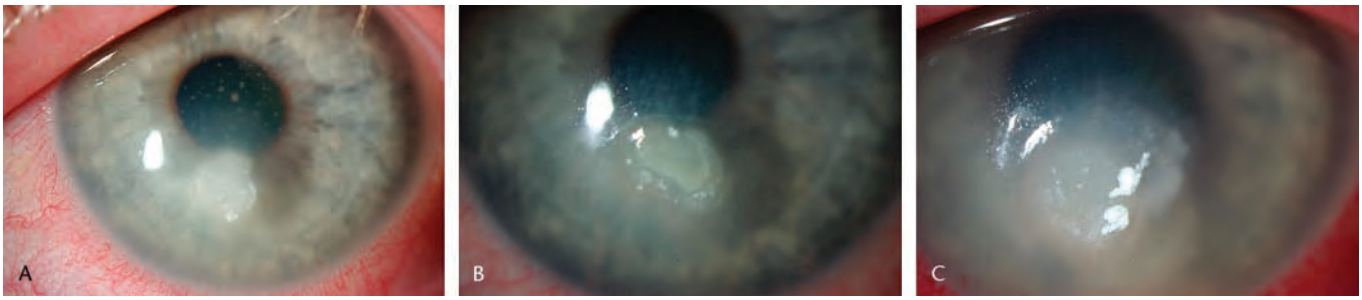

FIGURE 1. Sequential photographs of case 1 *B. bassiana* left corneal ulcer. Photographs taken on January 19, 2006 (A), March 30, 2006 (B), and April 6, 2006 (C).

A 2.3 × 3 mm epithelial plaque with an epithelial defect, mild stromal edema, and 1+ keratic precipitates remained (Fig. 1B).

Despite increasing the antifungal drops, over the next week, a severe corneal ulcer with impending perforation developed, requiring emergent penetrating keratoplasty with intravitreal vancomycin (Fig. 1C). Clinically, the infection appeared bacterial but the corneal specimen showed no fungal or bacterial organisms on periodic acid–Schiff and Gram stains. She did well after the keratoplasty with left cataract surgery 8 months later. On August 2, 2007, the graft was clear with intact sutures and the best-corrected visual acuity was 20/20.

On January 10, 2007, a 31-year-old healthy Caucasian woman was referred to the Department of Ophthalmology and Visual Sciences at the University of Wisconsin Hospitals and Clinics for a persistent soft contact lens–related corneal ulcer. Her right eye became red and painful 5 weeks earlier, and she was taking topical moxifloxacin, prednisolone acetate 1%, homatropine 5%, Polysporin ointment, and artificial tears. She wore Acuvue OAsys soft contact lenses cleansed with Bausch & Lomb ReNu, AQuify (CIBA Vision, Duluth, GA), and Opti-Free (Alcon, Inc, Fort Worth, TX) solutions. She denied sleeping in her contacts, ocular trauma, and foreign bodies, and she was not an outdoors enthusiast.

The visual acuity without correction was 20/400. A 4-mm feathery infiltrate with multiple superior white satellite lesions surrounded a right paracentral corneal ulcer with a 1 × 0.8 mm epithelial defect. Corneal scrapings were sent for smears and cultures. The moxifloxacin and prednisolone acetate drops were discontinued, and 15 mg/mL fortified gentamicin and 50 mg/mL vancomycin drops alternating every hour were begun with continued homatropine twice daily and Polysporin at night.

The next day, the microbiology laboratory reported filamentous mycelial fragments on the slide stained with calcofluor white. Vancomycin and gentamicin drops were stopped, and topical amphotericin and natamycin drops every hour were started. Two days after corneal scraping, she felt better and photographs were taken (Fig. 2A), but by day 5, the pain worsened and the visual acuity dropped to count fingers at 2 feet. The conjunctiva was hyperemic, the ulcer enlarged to 3 × 2.5 mm with +1 corneal edema, and fibrin with 3+ flare filled the anterior chamber. Given the increased infiltrate density and anterior chamber reaction, 200 mg oral voriconazole twice daily and moxifloxacin drops 4 times daily were prescribed.

Six days after scraping, a fungal culture isolate was identified as a *Beauveria* species. Over the next 10 days, the amphotericin and natamycin drops were tapered because the anterior chamber reaction subsided. Twelve days after scraping, the ulcer measured 1 × 2.2 mm and surrounding infiltrate measured 3 × 3.2 mm (Fig. 2B). By postscraping day 16, the visual acuity had improved to 20/100, the epithelial defect had healed, and the anterior chamber was clear. The amphotericin and moxifloxacin drops were discontinued. Oral voriconazole continued for a total of 4 weeks, and natamycin was tapered over 9 weeks. On postscraping day 29, the visual acuity was 20/60, the anterior chamber was quiet, and her eye finally felt better.

Morphologic features and DNA sequencing at the Fungus Testing Laboratory at the University of Texas Health Science Center at San Antonio confirmed *B. bassiana*. DNA sequencing of the internal transcribed spacer and domains 1 and 2 (D1/D2) regions showed the fungus was *Cordyceps bassiana*, the teleomorph, or sexually reproducing form of *B. bassiana*. Sixteen weeks after the initial consult, best-corrected visual acuity was 20/30. The corneal scar measured 2.5 × 2.5 mm with 10% stromal thinning (Fig. 2C). By October 22, 2007, the scar had faded and the best-corrected visual acuity was 20/25. An Orbscan confirmed moderate irregular astigmatism seen on keratometry readings.

Beauveria keratitis case reports listed in PubMed are summarized in Table 1.[5–10] Tu and Park[5] described a 58-year-old woman from northern Illinois who developed *B. bassiana* keratitis in 2007. She wore Acuvue II soft contact lenses cleansed with Opti-Free solutions. Initial culture and smears were negative, but confocal

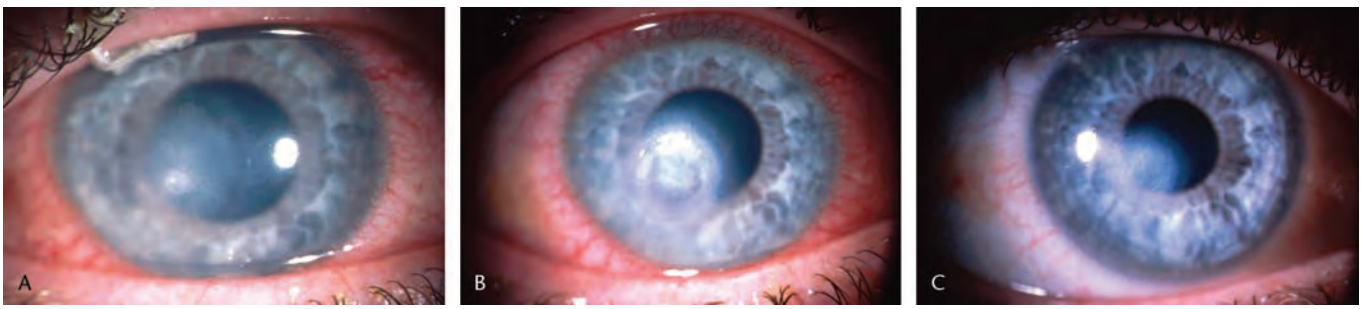

FIGURE 2. Sequential photographs of case 2 *B. bassiana* right corneal ulcer. Photographs taken on January 12, 2007 (A), January 22, 2007 (B), and May 4, 2007 (C).

TABLE 1. *Beauveria* Keratitis Cases Listed in PubMed

Author, Year, Reference (PubMed unique identifier)	Patient, Location, *Beauveria* Species	Diagnostic Method, Soft Contact Lens, Cleaning Solution(s)	Treatment	Outcome
Pariseau, 2010, current study	55-year-old diabetic woman, New Mexico, *B. bassiana*	Corneal culture, CooperVision Proclear Multifocal, Bausch & Lomb ReNu with MoistureLoc	Prednisolone and moxifloxacin drops before diagnosis, 200 mg oral ketoconazole twice daily, ketoconazole, natamycin, ketorolac tromethamine drops	Secondary bacterial infection, penetrating keratoplasty with intravitreal vancomycin, cataract extraction, best-corrected visual acuity 20/20 twenty months after initial evaluation
	31-year-old woman, southern Wisconsin, *B. bassiana*	Calcofluor white stain of corneal scrapings, corneal culture confirmed by DNA sequencing, Acuvue Oasys, ReNu, Aquify, Opti-Free	Moxifloxacin and prednisolone acetate 1% drops by referring optometrist, 15 g/mL gentamicin, and 50 mg/mL vancomycin drops for 1 d before diagnosis, natamycin, amphotericin, moxifloxacin, 200 mg oral voriconazole twice daily for 28 d	Residual corneal scar, best-corrected visual acuity 20/25 nine months after initial evaluation
Tu and Park[5] (17721308)	58-year-old woman, Illinois, *B. bassiana*	Confocal microscopy, Diff-Quick smear, corneal culture, Acuvue II, Opti-Free	Moxifloxacin, prednisolone, TobraDex/dexamethasone, scopolamine, trifluridine drops before evaluation, 14 mg/mL tobramycin and 50 mg/mL vancomycin for 3 d, natamycin 5%, topical sulfamethoxazole/trimethoprim, topical voriconazole, 200 mg/d oral fluconazole then 200 mg oral posaconazole 4 times/d for 1 mo	Elevated liver function tests and pleural effusions attributed to posaconazole; keratitis "judged to have completely healed" after 1 month of treatment
Kisla et al[10] (10832710)	82-year-old woman, Illinois, *B. bassiana*	Giemsa smear, cultures of dehisced corneal graft	Prednisolone acetate drops, gentamicin ointment before diagnosis, then natamycin 5% drops × 3 months, 200 mg oral fluconazole 2 times/d for 3 wk	Repeat penetrating keratoplasty for persistent corneal edema, uncorrected visual acuity 20/60 eleven months after surgery
Low et al[9] (9395884)	67-year-old woman, Australia, *B. bassiana*	Gram, Giemsa smears, cultures from lamellar dissection	Chloramphenicol drops, prednisone phosphate and atropine drops before diagnosis, then miconazole 1%, natamycin 5% drops	3 × 2-mm lamellar dissection of infected tissue; visual acuity 20/30 five months after hospitalization
Sachs et al[8] (2990527)	64-year-old man, Massachusetts, *B. bassiana*	Stains and cultures from scrapings negative; hematoxylin and eosin stain and cultures of corneal button	Gentamicin drops, chloramphenicol–polymyxin–hydrocortisone ointment, repeat gentamicin drops, dexamethasone ointment, repeat chloramphenicol–polymyxin–hydrocortisone ointment, atropine drops	Perforation sealed with isobutyl cyanoacrylate glue, followed by penetrating keratoplasty; "so far … best visual acuity 6/13 …"
McDonnell et al[6] (6100486)	70-year-old man, West Virginia, *B. alba*	Repeated scrapings and cultures were negative for fungus; Gomori methenamine stain of excised corneal stroma	Topical dexamethasone and mydriatics, topical and intravenous cefazolin and gentamicin, topical steroids, before diagnosis, then 200 mg oral ketoconazole 3 times daily, intravenous ketoconazole poorly tolerated, topical steroids	Penetrating keratoplasty, removal of superior iris, ciliary body, and sclera, repeat penetrating keratoplasty; patient did "well" during a 33-month follow-up period
Ishibashi et al[7] (6541434)	44-year-old man, Japan, *B. bassiana*	Potassium hydroxide and India ink stain, cultures	Antibiotic, corticosteroid drops before diagnosis, antibiotic drops, 24.6 g intravenous miconazole over 3 wk	Ulcer completely healed, best-corrected visual acuity 20/20

microscopy and a Diff-Quick (Difco Laboratories, Detroit, MI) stain of corneal scrapings showed filamentary forms. A repeat culture grew *Beauveria*, and topical natamycin 5%, topical sulfamethoxazole-trimethoprim, topical voriconazole 1%, 200 mg oral fluconazole daily followed by 1 month of 200 mg oral posaconazole 4 times daily were prescribed. Because of pleural effusions and elevated liver function tests, posaconazole was discontinued but the keratitis resolved.

MATERIALS AND METHODS

Four *B. bassiana* strains are registered with the Environmental Protection Agency.[11] They are sold as biopesticides marketed as organic or natural insect control. *B. bassiana* strain GHA was isolated from a beetle in 1977 and approved in 1995 for all food and feed crops and certain

nonfood uses. Manufactured by Laverlam International Corporation (Butte, MI) under the names BotaniGard and Mycotrol O, GHA is sold as a wettable powder or emulsified suspension.[12] *B. bassiana* American Type Culture Collection 74040 was approved for use on food and feed crops on May 3, 1999. It is sold under the name Naturalis by Troy Biosciences, Inc (Phoenix, AZ).[13] GlycoGenesys registered *B. bassiana* 447 as indoor bait for fire ants in a product called the Baits Motel Stay Awhile–Rest Forever, but the company went bankrupt. Jabb of the Carolinas (Pine Level, NC) registered *B. bassiana* HF23 on March 14, 2007, for manufacturing insecticides and balEnce, a spray to control flies in poultry houses whose chicken manure will be composted into crop fertilizer.[14]

To ascertain if our patients, and Tu and Park's patient, developed infection from a commercial *Beauveria* strain, we contacted Dr. Richard A. Humber, the curator of the US Department of Agriculture Agricultural Research Service Collection of Entomopathogenic Fungi (ARSEF).[15] Located in Ithaca, NY, and affiliated with Cornell University, this collection is the largest in the world.[16]*Beauveria* GHA is registered as accession number ARSEF 201, and *Beauveria* American Type Culture Collection 74040 is registered as ARSEF 3097. In exchange for samples of these strains, cultures of case 1 and case 2 were submitted to the collection as ARSEF 8601 and ARSEF 8602. The 3 corneal isolates were cultured on potato flakes agar and examined on microscopic slides stained with lactophenol cotton blue or lactofuchsin mounting medium. Because the isolates looked alike, they could be distinguished only by molecular characterization. Strain relatedness of the corneal and biopesticide isolates was determined by RAPD analysis using primers shown to differentiate *B. Bassiana*.[17]

RESULTS

Figure 3 is a photograph of a case 1 slide culture stained with lactophenol cotton blue at 10× magnification. All isolates looked identical, with septate hyaline hyphae and conidial production occurring in discrete masses. Figure 4 is a photograph of a case 2 slide culture stained with lactophenol cotton blue, showing a closer view of one of the many conidia clusters. For variety, Figure 5 is a photograph of Tu and Park's isolate stained with lactofuchsin mounting medium at 1000× magnification. It shows conidiogenous cells inflated at the base (white arrows), terminating in a denticulate geniculate rachis bearing apiculate, single-celled, globose, subglobose, and oval (black arrow) smooth conidia measuring 2–2.5 × 2–3 μm. These morphologic features of the corneal cultures were consistent with those published, and the isolates were identified as *B. bassiana*.[18,19] After 5 days incubation, the corneal isolates grew luxuriantly at 25°C but failed to grow at 35°C, a feature of a *B. bassiana* isolate causing disseminated disease in a patient with leukemia.[20] Figure 6 shows the RAPD polymerase chain reaction results. Each of the 5 isolates had a unique banding pattern, signifying that they are unrelated.

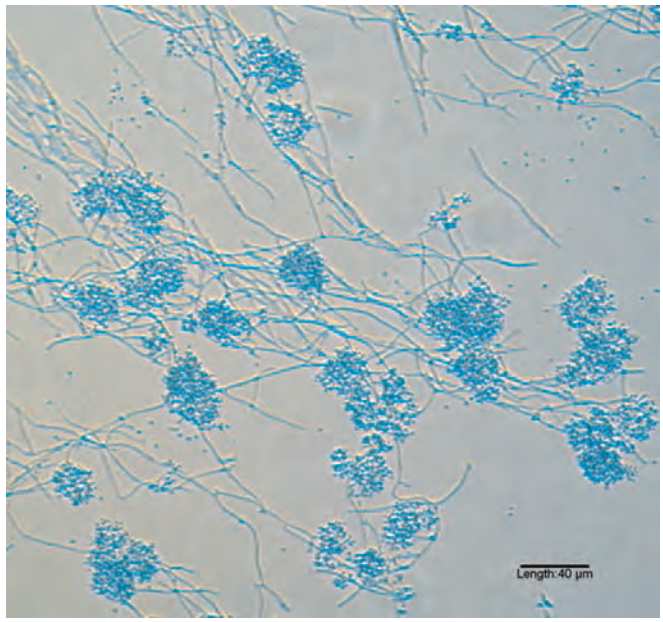

FIGURE 3. Microscopic slide culture of case 1 isolate stained with lactophenol cotton blue at 10× magnification. All corneal cultures looked alike and were prepared on potato flakes agar. The hyphae were septate and hyaline with conidiogenesis occurring in discrete masses along the hyphae. The scale is 40 μm.

DISCUSSION

Beauveria keratitis case reports listed in PubMed are summarized in Table 1.[5–10] McDonnell et al[6] described the only known case of *Beauveria alba* keratitis in 1985. Their

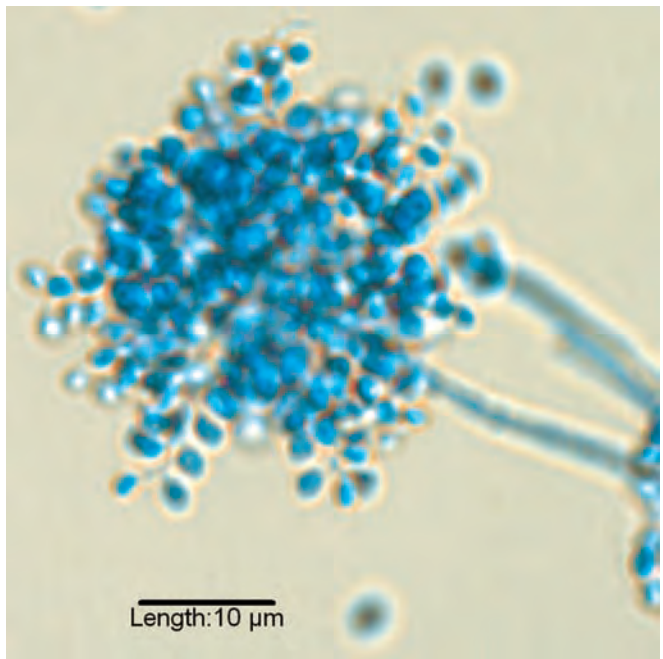

FIGURE 4. Microscopic slide culture of case 2 isolate stained with lactophenol cotton blue showing a closer view of one of the conidia clusters. The scale is 10 μm.

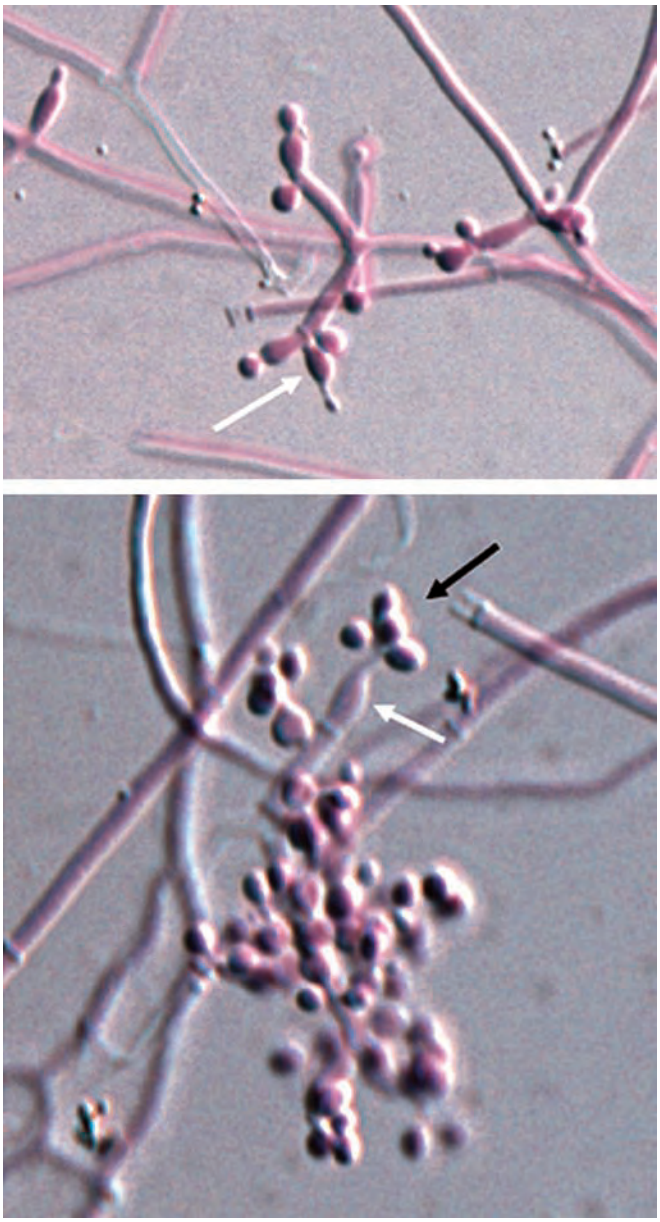

FIGURE 5. Microscopic slide culture of Tu and Park's isolate mounted in lactofuchsin mounting medium (magnification 1000×). For all isolates, the conidiogenous cells occurred in clusters, had inflated bases (white arrows), and terminated in a denticulate geniculate rachis bearing apiculate, single-celled, globose, subglobose, and oval (black arrow) smooth conidia measuring 2–2.5 × 2–3 μm.

patient was a 70-year-old man from West Virginia who developed mycotic keratitis 2 months after phacoemulsification and intraocular lens implantation. He was treated with antibiotics and steroids, but because of impending perforation underwent penetrating keratoplasty.

Although prior articles credit Sachs et al with reporting the first *B. bassiana* keratitis case, Ishibashi et al[7] reported *B. bassiana* keratitis in 1984. Their patient was a 44-year-old man in Japan who developed a left corneal ulcer from a dust particle. He was treated with 24.6 g intravenous miconazole over 3 weeks. The ulcer healed with a best-corrected visual acuity of 20/20.

Sachs et al[8] reported a *B. bassiana* keratitis case in 1985. The patient was a 64-year-old Caucasian Massachusetts farmer who sustained a right corneal abrasion with vegetable matter in October 1980. The matter was removed, but despite topical treatment with antibiotics and corticosteroids, the cornea perforated, requiring penetrating keratoplasty.

The third case of *B. bassiana* keratitis was reported in 1997 by Low et al.[9] Their patient was a 67-year-old woman in Australia whose infection surrounded a presumed caterpillar hair lodged deep in the cornea. As with the 2 prior cases, initial treatment with topical antibiotics and corticosteroids proved inadequate and required a 3- × 2-mm lamellar dissection.

Kisla et al[10] reported the fourth *B. bassiana* keratitis case in 2000. The patient was an 82-year-old Illinois woman who sustained blunt trauma to her 1989 corneal graft in June 1997. She underwent anterior vitrectomy and surgical repair of inferior graft dehiscence, with suture removal in December 1997. One month later, she developed graft edema, an epithelial defect, and a stromal infiltrate that was treated with topical prednisolone acetate and then gentamicin. Once smears and cultures showed fungal infection, topical natamycin 5% hourly and 200 mg oral fluconazole twice daily for 3 weeks was prescribed. Persistent corneal edema necessitated a second penetrating keratoplasty in April 1998.

Tu and Park[5] reported the fifth *B. bassiana* keratitis case in 2007, as described earlier. Two additional *B. bassiana* keratitis cases from Korea and 1 from Japan were found using Google, but none of them were listed in PubMed. One from Korea is a downloadable Power Point presentation given at the American Society of Cataract and Refractive Surgery 2008 meeting describing a 70-year-old man who injured his left cornea with a tree branch.[21] Despite treatment with natamycin drops, the cornea perforated, requiring amniotic membrane grafting and amphotericin drops, with the patient awaiting penetrating keratoplasty.[22] The other Korean case (only the abstract and figure captions are in English) reports a 64-year-old man with a 10-year history of herpetic keratitis that was treated with voriconazole (unknown if topical or oral), eventually requiring penetrating keratoplasty.[23] The case report from Japan is of an 80-year-old woman treated at the Ideta Eye Hospital.[24] She struck her left eye with the frame of her glasses, developed a corneal ulcer, and was treated with topical 0.1% miconazole, topical 1% voriconazole every hour, 1% pimaricin ointment daily, and 100 mg/d oral itraconazole. In these 3 cases, neither the final outcome nor visual acuity is known.

There have been only 2 reports of systemic *B. bassiana* infection; both occurred in immunosuppressed patients with leukemia. The first was a 38-year-old woman with acute myeloid leukemia who developed an allergic alveolitis with hepatic and splenic lesions.[24] The second was a 44-year-old woman diagnosed with acute lymphoblastic leukemia with disseminated skin lesions invading blood vessels and a hemorrhagic pleural effusion that was "consistently sterile."[20] In each case, the authors postulated that airborne spores caused pulmonary infection that spread hematogenously. This correlates with a case report from Turkey

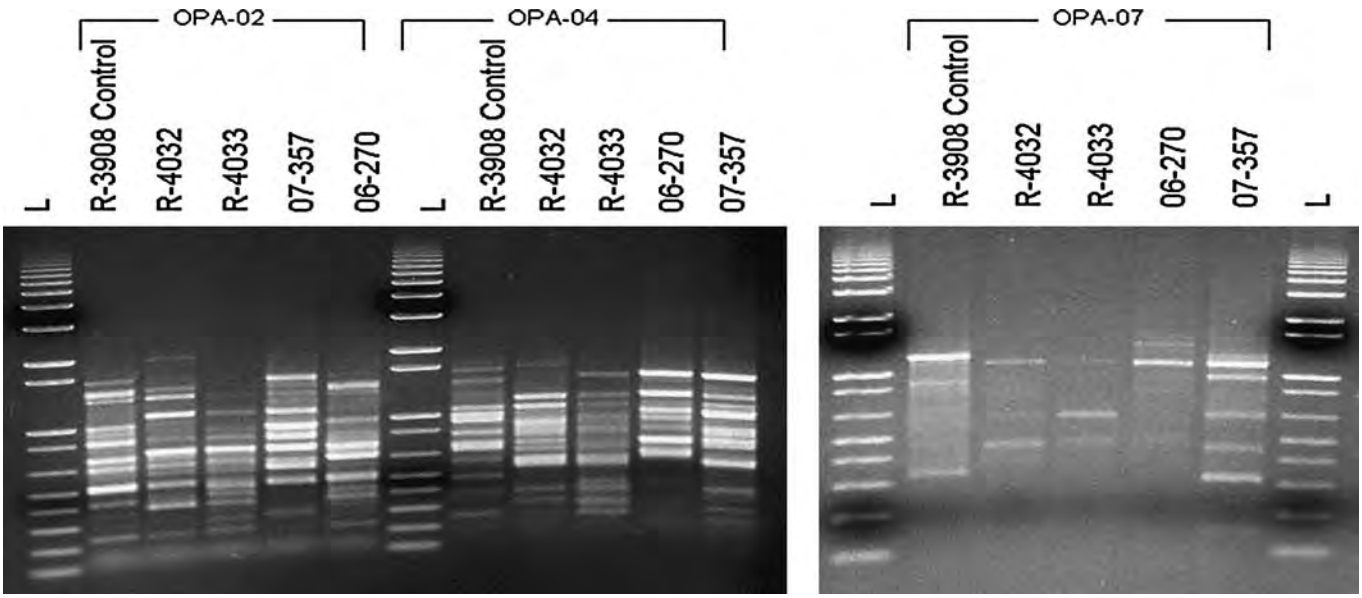

FIGURE 6. RAPD comparison of the 5 *B. bassiana* isolates using 3 different primers OPA-02, OPA-04, and OPA-07. The case 2 isolate was the control (R-3908). Case 1 = 06-270 and the case of Tu and Park = 07-357. The 2 biopesticides *Beauveria* GHA and *Beauveria* ATCC 74040 are labeled R-4032 and R-4033. Banding patterns for each isolate are distinct, indicating that the isolates are unrelated.

describing a patient who underwent a thoracic wall resection with lobectomy and developed a postoperative empyema attributed to *B. bassiana*.[25]

Beauveria and other fungi are used as biopesticides worldwide. In a review article about human exposure to fungi used in biocontrol agents, Madsen et al[1] report that *Beauveria* has been found in indoor environments, is often isolated from insects and soil samples, and because conidia are presumably dispersed by air currents, it may occur in air where insect hosts live. With multiple reports of increased prevalence of soft contact lens–related microbial keratitis, determining the etiology is important. Unlike earlier *B. bassiana* keratitis cases, all occurring after corneal injury in patients who did not wear contact lenses, our 2 patients, and Tu and Park's patient, wore soft contact lenses and had no history of ocular trauma. In a chart review at the University of Florida, Iyer et al[26] found that the percent of fungal ulcers caused by nontherapeutic contact lenses increased from 21% between 1999 and 2001 to 32% in 2002–2004, to 45% in 2005–2006, and after 2005, contact lens use surpassed trauma as the most common risk factor. Possible causes included an increasing number of contact lens wearers, younger patients who may be non-compliant, overnight wear, improper lens care, and a shift from thermal disinfection systems and peroxide-based solutions to multiuse-based solutions. Ide et al[27] analyzed ReNu MultiPlus, ReNu with MoistureLoc, and OPTI-FREE RepleniSH and concluded that the antifungal activity of contaminated multipurpose solutions may be insufficient to prevent contact lens fungal colonization. With voluntary recalls of Bausch & Lomb's ReNu with MoistureLoc in May 2006 for association with *Fusarium* keratitis and Advanced Medical Optics Complete MoisturePlus (Santa Ana, CA) in May 2007 for association with *Acanthamoeba* keratitis, we considered contaminated solutions or contact lens cases as possible infection sources. For case 1, the contact lens brand and cleaning solution were verified with the patient who easily recounted what she used. By the time she was referred, the contact lenses and cleaning solution were no longer available. In case 2, the contact lens brand and cleaning solutions were verified with the referring optometrist and the patient. We draw no conclusions about associations between *Beauveria* keratitis and specific contact lens brands or cleaning solutions.

Our study indicates that the organisms from these recent cases are unrelated to one another and to *Beauveria*-based biopesticides sold in the United States. It is interesting that although *Beauveria* is spread worldwide, 3 of the 6 reported *Beauveria* keratitis cases within the United States occurred in the Illinois–Wisconsin region, and our second case and Tu and Park's case occurred simultaneously in close geographic proximity. Our second case differs from prior reports in some aspects. She was 1 of 2 *Beauveria* keratitis cases confirmed with DNA sequencing and the first to be treated with oral voriconazole. She was 13–51 years younger than all other reported cases, which may have helped healing and one of the few cases that did not require penetrating keratoplasty or lamellar dissection. She denied any history of eye surgery, trauma, or foreign bodies, unlike the earlier cases listed in Table 1.

Our cases are similar to other *Beauveria* keratitis reports in that all patients were initially treated with topical antibacterials and corticosteroids before diagnosis without improvement. We agree with authors of prior *Beauveria* keratitis cases who commented that steroids and antibiotics may have caused fungal growth. Similar to other fungal keratitis cases treated with oral voriconazole, our second patient healed and did not require a penetrating keratoplasty.[28,29] There are reports of

worsening fungal keratitis when treated only with topical 1% voriconazole.[30,31] Three small case series measured aqueous concentrations at different times after various topical 1% voriconazole dosing regimens; one study specifically sampled during troughs and rinsed the eye before sampling.[32–34] The measured voriconazole concentrations were variable. A 2008 Cochrane Review of medical interventions for fungal keratitis concluded that the first line of treatment in fungal keratitis is topical antifungal agents, but "there is no evidence that current available and investigational antifungal agents are effective."[35] In contrast, a 2008 voriconazole review article concluded that oral voriconazole may be considered as first-line therapy for fungal keratitis.[36]

CONCLUSION

Using RAPD analysis, this study shows that although the 3 most recent *Beauveria* keratitis cases all wore soft contact lenses, their isolates are unrelated to one another and to *Beauveria*-based biopesticides sold in the United States. The difference in associated cause between older and current *Beauveria* cases matches the change in associated cause for fungal keratitis in general; older cases are associated with corneal injury, particularly with plant matter, whereas newer cases occur in contact lens wearers. The etiology of these newer cases is unknown, and one wonders if they represent a true increase in fungal keratitis or heightened awareness of microbial keratitis. By storing the isolates at the Agricultural Research Service Collection of Entomopathogenic Fungi and the Fungus Testing Laboratory of the University of Texas Health Science Center at San Antonio, we hope to aid future researchers.

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Sequence-Based Identification of Filamentous Basidiomycetous Fungi from Clinical Specimens: a Cautionary Note▽

Anna M. Romanelli,[1] Deanna A. Sutton,[2] Elizabeth H. Thompson,[2] Michael G. Rinaldi,[2] and Brian L. Wickes[1]*

Department of Microbiology and Immunology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas,[1] *and Department of Pathology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas*[2]

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The species-level identification of sterile and/or arthroconidium-forming filamentous fungi presumed to be basidiomycetes based upon morphological or physiological features alone is usually not possible due to the limited amount of hyphal differentiation. Therefore, a reliable molecular approach capable of the unambiguous identification of clinical isolates is needed. One hundred sixty-eight presumptive basidiomycetes were screened by sequence analysis of the internal transcribed spacer (ITS) and D1/D2 ribosomal DNA regions in an effort to obtain a species identification. Through the use of this approach, identification of a basidiomycetous fungus to the species level was obtained for 167/168 of the isolates. However, comparison of the BLAST results for each isolate for both regions revealed that only 28.6% (48/168) of the isolates had the same species identification by use of both the ITS and the D1/D2 regions, regardless of the percent identity. At the less stringent genus-only level, the identities for only 48.8% (82/168) of the isolates agreed for both regions. Investigation of the causes for this low level of agreement revealed that 14% of the species lacked an ITS region deposit and 16% lacked a D1/D2 region deposit. Few GenBank deposits were found to be complete for either region, with only 8% of the isolates having a complete ITS region and 10% having a complete D1/D2 region. This study demonstrates that while sequence-based identification is a powerful tool for many fungi, sequence data derived from filamentous basidiomycetes should be interpreted carefully, particularly in the context of missing or incomplete GenBank data, and, whenever possible, should be evaluated in light of compatible morphological features.

The emergence of rare but clinically significant fungi has placed a growing diagnostic burden on clinical microbiologists. Nevertheless, the accurate identification of these etiologic agents remains critically important, despite the low frequency of some species that are encountered in clinical specimens (10, 21). For filamentous fungi, identification by the use of colonial and microscopic morphologies, the major identification method, largely depends on the production of reproductive structures. Although filamentous basidiomycetes rarely cause disease, they are increasingly recognized from clinical specimens (27). However, definitive identification can be problematic, with many isolates remaining sterile in culture (15, 23, 28). The inability to ascertain a genus or species due to the lack of observable reproductive structures can potentially increase the time to the reporting of an inconclusive result and, consequently, adversely affect treatment strategies (13, 26, 29). Therefore, there is a clear need for alternative methods for the identification of fungi that do not produce morphologically distinguishing features.

Sequencing of the ribosomal genes has emerged as a useful diagnostic tool for the rapid detection and identification of fungi, regardless of whether morphologically distinct structures are produced (6, 16, 32). One of the most common ribosomal targets for sequence identification is the internal transcribed spacer (ITS) region. This region contains two informative regions, ITS1 and ITS2, which are located between the 18S and 28S ribosomal subunits and which are separated by the 5.8S ribosomal subunit (8, 9). The ITS region can be amplified from a broad spectrum of fungi with primers ITS-1 and ITS-4 and can generally be recovered in a single PCR, since the amplicon is usually ~400 to 700 bp in length (9, 11, 17). A second variable site within the ribosomal DNA (rDNA) cluster, called the D1/D2 region, can also be amplified from a broad spectrum of fungi with primers NL-1 and NL-4, although it is usually less variable than the ITS region (19). The D1/D2 region is located toward the 5′ end of the large ribosomal subunit (26S or 28S) and overlaps the ITS region at the ITS-4/NL-1 primer site. The combination of conserved and variable regions offers great flexibility for PCR sensitivity and specificity. The conserved sequences at the flanking ends of the D1/D2 and ITS regions provide universal PCR priming sites, while the variable internal regions provide species-specific sequences in many cases (4, 7, 19).

Although the ITS region displays enough sequence variability to allow the identification of many fungi to the species level, for some fungi the sequence of the ITS region alone is not sufficient for accurate identification to the species level (1, 24). In these cases, a second locus, such as that for β-tubulin or calmodulin, can be sequenced (2, 3). Unfortunately, universal priming sites, which are required to obtain an amplicon from an unknown fungus, are sacrificed for the more variable nature of these nonribosomal genes, which in turn requires enough knowledge of the strain identity to allow the selection of primers that will yield a PCR product. Since sterile molds could potentially be found in any phylum, it would not be possible to

* Corresponding author. Mailing address: Department of Microbiology and Immunology, The University of Texas Health Science Center at San Antonio, Mail Code 7758, Room 5.027V, 7703 Floyd Curl Dr., San Antonio, TX 78229-3900. Phone: (210) 567-3938. Fax: (210) 567-6612. E-mail: wickes@uthscsa.edu.

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TABLE 1. Strains used in this study

Strain no.	Accession no.	Yr[a]	Phenotypic features[b]	Source	H, A, or O source[c]
B-1	07-56	2007	Sterile, chlamydoconidia, crystal-encrusted hyphae	BAL[d]	H
B-2	07-31	2007	Sterile, crystals	BAL	H
B-3	06-4454	2006	Arthroconidia	Eye	H
B-4	06-4450	2006	Arthroconidia	Scalp	H
B-5	06-4444	2006	Arthroconidia	BAL	H
B-6	06-4410	2006	Sterile, chlamydoconidia	BAL	H
B-7	06-4341	2006	Sterile, chlamydoconidia	Sputum	H
B-8	06-4285	2006	Sterile, chlamydoconidia	BAL	H
B-9	06-4161	2006	Arthroconidia	Neck mass	H
B-10	06-4137	2006	Sterile	BAL	H
B-11	06-4124	2006	Sterile	BAL	H
B-12	06-4103	2006	Arthroconidia, crystals	Bronchial wash	H
B-13	06-4057	2006	Sterile, mushroom smell	Sputum	H
B-14	06-3994	2006	Sterile, crystals, spathulate hyphae	Nasal	H
B-15	06-3970	2006	Arthroconidia, brown diffusing pigment	BAL	H
B-16	06-3924	2006	Arthroconidia	BAL	H
B-17	06-3906	2006	Sterile, crystal-encrusted hyphae	Sputum	H
B-18	06-3888	2006	Arthroconidia	BAL	H
B-19	06-3869	2006	Sterile	Aspirate	H
B-20	06-3821	2006	Arthroconidia, chlamydoconidia	Bronchial wash	H
B-21	06-3806	2006	Sterile	Sputum	H
B-22	06-3795	2006	Arthroconidia	Bronchial wash	H
B-23	07-312	2007	Arthroconidia, chlamydoconidia	Axillary and lymph nodes	A
B-24	06-3621	2006	Arthroconidia	BAL	H
B-25	06-3536	2006	Curved conidia, gold	BAL	H
B-26	06-3497	2006	Sterile	BAL	H
B-27	06-3349	2006	Arthroconidia	Lung	H
B-28	06-3341	2006	Arthroconidia	BAL	H
B-29	06-3321	2006	Curved conidia, gold	BAL	H
B-30	07-315	2007	Sterile	Draining tract	A
B-31	06-3788	2006	Sterile, chlamydoconidia	BAL	H
B-32	06-3787	2006	Sterile, chlamydoconidia	Lung	H
B-33	06-3769	2006	Arthroconidia	BAL	H
B-34	06-3768	2006	Sterile	Lung	H
B-35	06-3499	2006	Sterile	BAL	H
B-36	06-3466	2006	Sterile, chlamydoconidia	BAL	H
B-37	06-3460	2006	Sterile, chlamydoconidia	BAL	H
B-38	06-3335	2006	Sterile	BAL	H
B-39	06-3320	2006	Sterile, chlamydoconidia	BAL	H
B-40	05-1243	2005	Arthroconidia	BAL	H
B-41	05-1416	2005	Arthroconidia, crystal-encrusted hyphae	Sputum	H
B-42	05-2219	2005	Curved conidia	BAL	H
B-43	05-2239	2005	Arthroconidia, chlamydoconidia	BAL	H
B-44	05-2353	2005	Spicules	Sinus	H
B-45	05-2587	2005	Arthroconidia, chlamydoconidia	BAL	H
B-46	05-2369	2005	Sterile	BAL	H
B-47	07-551	2007	Sterile	BAL	H
B-48	07-495	2007	Sterile, chlamydoconidia, gold-brown	BAL	H
B-49	05-567	2005	Sterile, setal hyphae	Carapace	A
B-50	05-459	2005	Curved conidia	Aortic graft tissue	H
B-51	05-597	2005	Clamp connections, crystals	BAL	H
B-52	05-679	2005	Sterile, chlamydoconidia	BAL	H
B-53	05-1037	2005	Sterile, chlamydoconidia	BAL	H
B-54	05-1063	2005	Sterile, chlamydoconidia, crystals	BAL	H
B-55	05-1422	2005	Sterile, crystals	Tissue	A
B-56	05-1553	2005	Curved conidia	BAL	H
B-57	05-1560	2005	Sterile, crystals	BAL	H
B-58	05-1575	2005	Sterile, chlamydoconidia, crystals	Right lower lung tissue	H
B-59	05-1822	2005	Sterile	BAL	H
B-60	05-1853	2005	Arthroconidia, chlamydoconidia, crystals	BAL	H
B-61	05-1932	2005	Sterile	Lung tissue	H
B-62	05-2034	2005	Sterile	Respiratory	A
B-63	05-2061	2005	Arthroconidia, chlamydoconidia	BAL	H
B-64	05-2112	2005	Arthroconidia	Sputum	H
B-65	05-2164	2005	Sterile, crystals	Sputum	H
B-66	05-2269	2005	Arthroconidia, clamp connections	BAL	H

Continued on following page

TABLE 1—*Continued*

Strain no.	Accession no.	Yr[a]	Phenotypic features[b]	Source	H, A, or O source[c]
B-67	05-2308	2005	Sterile	Sputum	H
B-68	05-2341	2005	Sterile	BAL	H
B-69	05-2354	2005	Sterile	BAL	H
B-70	05-2474	2005	Sterile, crystals	BAL	H
B-71	05-2504	2005	Arthroconidia	Cranium	H
B-72	05-2586	2005	Sterile, crystals, yellow refractile hyphae	BAL	H
B-73	05-2588	2005	Sterile, crystals	BAL	H
B-74	05-2641	2005	Sterile, chlamydoconidia	BAL	H
B-75	06-3310	2006	Sterile	BAL	H
B-76	06-3308	2006	Sterile	BAL	H
B-77	06-3298	2006	Sterile, frequently septate hyphae	BAL	H
B-78	06-3297	2006	Arthroconidia	Sputum	H
B-79	06-3281	2006	Arthroconidia	Sinus	H
B-80	06-3259	2006	Arthroconidia	BAL	H
B-81	06-3223	2006	Arthroconidia	BAL	H
B-82	06-3212	2006	Curved conidia	BAL	H
B-83	06-3194	2006	Sterile	CSF	H
B-84	06-3190	2006	Sterile, skelatoid hyphae	BAL	H
B-85	06-3183	2006	Sterile	BAL	H
B-86	06-3182	2006	Arthroconidia	Bronchial biopsy	H
B-87	06-3176	2006	Sterile	BAL	H
B-88	06-3159	2006	Sterile	BAL	H
B-89	06-3094	2006	Sterile	Sputum	H
B-90	06-3093	2006	Sterile	BAL	H
B-91	06-3082	2006	Sterile, crystals, yellow-orange	BAL	H
B-92	06-3080	2006	Sterile	BAL	H
B-93	05-2738	2005	Sterile, chlamydoconidia, crystals, brown	Sputum	H
B-94	05-2742	2005	Basidiospores, phototropic	Pleural fluid	H
B-95	05-2777	2005	Arthroconidia, chlamydoconidia, crystals	Sputum	H
B-96	05-2954	2005	Sterile, crystals	BAL	H
B-97	05-3058	2005	Sterile	BAL	H
B-98	05-3255	2005	Sterile, chlamydoconidia	BAL	H
B-99	05-3313	2005	Arthroconidia	BAL	H
B-100	05-3368	2005	Sterile	BAL	H
B-101	05-738	2005	Sterile, refractile brown hyphae, crystals	Right wrist	H
B-102	05-2582	2005	Sterile, setal hyphae	BAL	H
B-103	05-2585	2005	Arthroconidia	BAL	H
B-104	07-729	2007	Sterile, chlamydoconidia	Urine	H
B-105	07-793	2007	Arthroconidia	BAL	H
B-106	07-797	2007	Arthroconidia	Sputum	H
B-107	05-2661	2005	Sterile, skelatoid hyphae, crystals	BAL	H
B-108	05-2677	2005	Sterile, crystals, clamp connections	Sputum	H
B-109	05-3095	2005	Arthroconidia, golden brown	BAL	H
B-110	05-3281	2005	Arthroconidia	BAL	H
B-111	07-864	2007	Arthroconidia	Lung wash	H
B-112	07-865	2007	Arthroconidia	BAL	H
B-113	07-866	2007	Arthroconidia	BAL	H
B-114	07-1061	2007	Sterile, crystal-encrusted hyphae	BAL	H
B-115	07-1076	2007	Arthroconidia	BAL	H
B-116	07-1092	2007	Arthroconidia, chlamydoconidia	Sputum	H
B-117	07-1095	2007	Arthroconidia	BAL	H
B-118	06-2442	2006	Arthroconidia	BAL	H
B-119	06-2441	2006	Produces arthroconidia	BAL	H
B-120	06-2439	2006	Arthroconidia, chlamydoconidia	BAL	H
B-121	06-2433	2006	Sterile	Sputum	H
B-122	06-2432	2006	Sterile, achanthohyphidia	BAL	H
B-123	06-2422	2006	Sterile, chlamydoconidia, crystals	BAL	H
B-124	06-2420	2006	Sterile, chlamydoconidia	BAL	H
B-125	06-2401	2006	Sterile	Sputum	H
B-126	06-2362	2006	Sterile, chlamydoconidia	Sputum	H
B-127	06-2358	2006	Sterile	BAL	H
B-128	06-2354	2006	Sterile	BAL	H
B-129	06-2341	2006	Sterile	BAL	H
B-130	06-2304	2006	Sterile	BAL	H
B-131	06-2581	2006	Arthroconidia, crystal-encrusted hyphae	Right foot	H
B-132	06-2571	2006	Arthroconidia	Sputum	H
B-133	06-2563	2006	Sterile	BAL	H

Continued on following page

TABLE 1—*Continued*

Strain no.	Accession no.	Yr[a]	Phenotypic features[b]	Source	H, A, or O source[c]
B-134	06-2544	2006	Sterile	BAL	H
B-135	06-2552	2006	Sterile	BAL	H
B-136	06-2544	2006	Sterile	Unknown	H
B-137	06-2536	2006	Arthroconidia, chlamydoconidia, conidia	Lung	H
B-138	06-2486	2006	Sterile	BAL	H
B-139	06-2736	2006	Curved conidia	BAL	H
B-140	06-2734	2006	Sterile	BAL	H
B-141	06-2729	2006	Curved conidia	BAL	H
B-142	06-2725	2006	Arthroconidia	BAL	H
B-143	06-2723	2006	Sterile	BAL	H
B-144	06-2721	2006	Sterile	BAL	H
B-145	06-2687	2006	Sterile	BAL	H
B-146	06-2685	2006	Sterile, clamp connections	BAL	H
B-147	06-2683	2006	Arthroconidia	BAL	H
B-148	06-2670	2006	Sterile, chlamydoconidia	BAL	H
B-149	06-2650	2006	Arthroconidia	Cornea	H
B-150	06-2644	2006	Curved conidia	BAL	H
B-151	06-2641	2006	Sterile, clamp connections	BAL	H
B-152	06-2629	2006	Sterile	Cornea	H
B-153	06-2624	2006	Sterile	BAL	H
B-154	06-3057	2006	Arthroconidia	BAL	H
B-155	06-3035	2006	Sterile	BAL	H
B-156	06-3002	2006	Sterile	BAL	H
B-157	06-3001	2006	Sterile, chlamydoconidia	BAL	H
B-158	06-2997	2006	Arthroconidia	BAL	H
B-159	06-2951	2006	Arthroconidia	BAL	H
B-160	06-2949	2006	Sterile, chlamydoconidia, orange	BAL	H
B-161	06-2947	2006	Arthroconidia	BAL	H
B-162	06-2939	2006	Sterile, chlamydoconidia	BAL	H
B-163	06-2860	2006	Sterile, chlamydoconidia	BAL	H
B-164	06-2839	2006	Arthroconidia	BAL	H
B-165	06-2833	2006	Sterile, chlamydoconidia	Sinus fluid	H
B-166	06-2807	2006	Arthroconidia	BAL	H
B-167	07-1060	2007	Sterile	Sputum	H
B-168	07-1074	2007	Sterile, spicules	Left sinus	H

[a] Year accessioned into the Fungus Testing Laboratory culture collection.
[b] Determined by growth on potato flakes agar at 25°C.
[c] H, human source; A, animal source; O, other source (e.g., the environment).
[d] BAL, bronchoalveolar lavage.

know for sure which gene-specific primer pair to select for use in a PCR assay, since the priming sites could be genus specific. Additionally, it is possible that sequencing of a second site could be even less informative than rDNA sequencing due to the fewer GenBank deposits for the target locus. Therefore, the goal of this study was to determine if combined sequencing of the ITS and D1/D2 regions of a large collection of mostly sterile filamentous molds, presumed to be basidiomycetes, could confirm this preliminary placement in the phylum *Basidiomycota* as well as provide an accurate species-level identification.

MATERIALS AND METHODS

Strains and media. The isolates that were used in this study were from a large collection archived in the Fungus Testing Laboratory (http://strl.uthscsa.edu/fungus/) in the Department of Pathology at the University of Texas Health Science Center at San Antonio (UTHSCSA) (Table 1). The isolates were maintained on potato dextrose agar (PDA; Difco, Detroit, MI) slants and had previously been identified as probable basidiomycetes on the basis of their macroscopic morphology on potato flakes agar (25), their microscopic features (noted in Table 1), and their physiological features. All isolates demonstrated rapid, woolly growth that was white to cream or golden, had the ability to grow on agar containing 10 μg/ml benomyl (30), and failed to grow on medium containing 0.5 μg/ml cycloheximide (Mycobiotic agar; Remel, Inc., Lenexa, KS). These candidate isolates were plated onto PDA, grown at 25°C for 4 to 7 days, and then submitted for molecular characterization.

DNA preparation. The isolates were again subcultured onto PDA and were grown for 24 h at 30°C. DNA was isolated from the hyphae by use of the Prepman Ultra reagent (Applied Biosystems, Foster City, CA), in which a small amount of material (enough to fill a loop) from each isolate was suspended in 50 μl of Prepman Ultra reagent in a 0.5-ml microcentrifuge tube. The suspension was initially vortexed for 45 s to 60 s to disperse the hyphal material and was then heated for 15 min at 100°C. The suspension was vortexed briefly and was then pelleted for 5 min at a maximum speed of 16,000 × *g* in a microcentrifuge. The supernatant was transferred to a new tube and stored on ice until the PCRs could be set up (within 1 h).

PCR. PCR was performed with a 50-μl volume, which contained the following: 3 μl of template DNA, 5 μl 10× PCR buffer, 5 μl of a 10 μM stock solution of each primer (ITS-1 forward primer [32] and NL-4 reverse primer [17, 19]), 1.5 μl of 10 mM deoxynucleoside triphosphates (Invitrogen, Carlsbad, CA), and 5.0 U of Triplemaster *Taq* DNA polymerase (Eppendorf, Westbury, NY). The PCRs were performed in an Eppendorf master thermocycler and were run with a temperature profile of 2 min at 94°C, followed by 35 cycles of 20 s at 94°C, 20 s at 60°C, and 1 min at 72°C. The 35 cycles were followed by 5 min at 72°C. A 5-μl aliquot of each PCR product and a negative no-DNA control were run on a 0.7% agarose gel, stained with ethidium bromide, and documented with a DC 290 imaging system (Eastman Kodak Co., Rochester, NY) to confirm that amplification took place. The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA), and both strands were sequenced through the original ITS-1 and NL-4 PCR primer sites. The sequences were

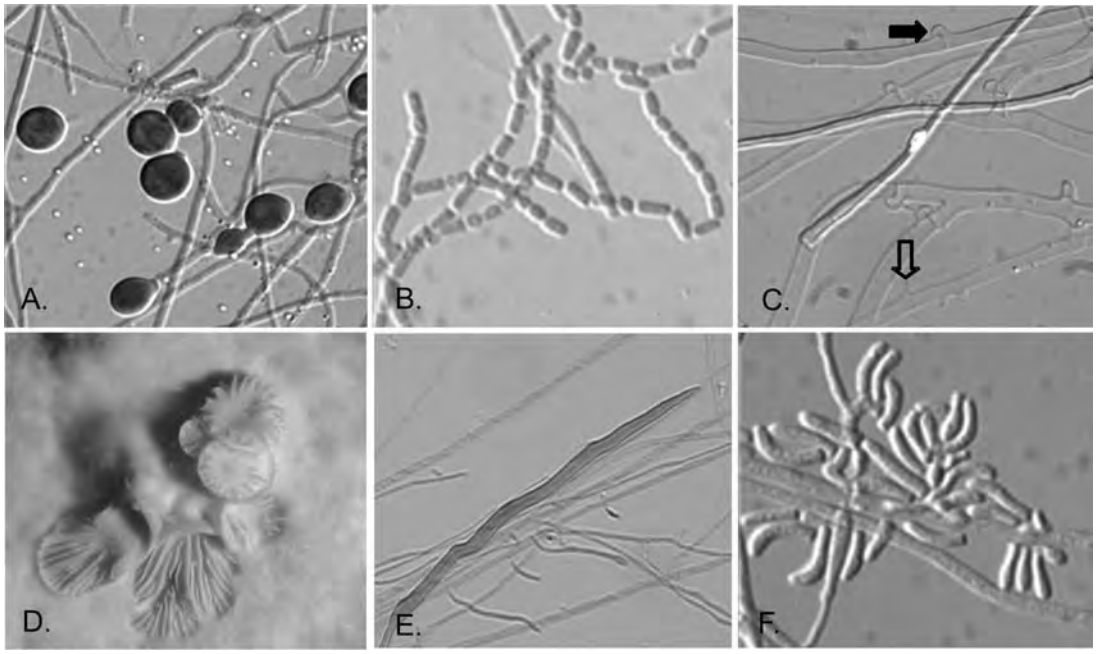

FIG. 1. Typical morphological features of basidiomycetes in culture. The morphological features that basidiomycetes may display in culture are shown. Microscopic features include chlamydoconidia (A), arthroconidia (B), spicules (open arrow) and clamp connections (solid arrow) of *Schizophyllum commune* (C), macroscopic basidiocarp of a dikaryotic *Schizophyllum commune* isolate (D), setal hyphae of *Inonotus* (*Phellinus*) *tropicalis* (E), and conidia of the *Hormographiella* anamorph of a *Coprinus* sp. (F). (A, B, C, E, F) Magnifications, ×880; (D) magnification, ×5.

obtained as overlapping runs of the two flanking primers (primers ITS-1 and NL-4), as well as runs of two internal primers (primers ITS-4 and NL-1) (9, 17, 32). Sequencing was performed at the UTHSCSA Advanced Nucleic Acids Core Facility, and data were obtained with Sequencing Analysis Software (version 5.3.1; Applied Biosystems).

Sequence analysis. The sequence data were assembled and analyzed by the use of MacVector software (MacVector, Inc., Cary, NC) and were then searched by using the ITS-1 and ITS-4 primer sequences to delineate the ITS region, as well as the NL-1 and NL-4 sequences to delineate the D1/D2 region. Each sequence was parsed into both the ITS and the D1/D2 regions and was then separately used to perform individual nucleotide-nucleotide searches with the BLASTn algorithm at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). The outputs from the BLAST searches were sorted on the basis of the maximum identity and were recorded as they appeared without modification of genus or species names that may have been synonyms or teleomorphs of other genus or species names in other GenBank records. Sequence-based identities with a cutoff of 97% or greater were considered significant in this study, and the best hit was defined as the sequence with the highest maximum identity to the query sequence.

RESULTS

Morphological basidiomycete identification. The isolates used in the study were identified as probable or presumptive basidiomycetes on the basis of their macroscopic, microscopic, and physiological features. Although a limited number of features of filamentous basidiomycetes are not diagnostic, they are suggestive for placement of the isolates in the phylum *Basidiomycota*. Growth is typically rapid, often up the side of the tube or plate; and colony colors are usually white, but they are sometimes cream to golden, orange, or slightly brownish on PDA. Microscopically, sterile basidiomycetes may display hyphae only or hyphae with chlamydoconidia (Fig. 1A). Some basidiomycetes do, however, produce conidia in culture. Most are arthroconidia, as seen in Fig. 1B, or compact clusters of arthroconidia, as seen for some *Hormographiella* species (anamorphs of some *Coprinopsis* [*Coprinus*] species). One of the more useful microscopic features for the identification of sterile isolates as basidiomycetes is the production of clamp connections, the defining characteristic for this phylum (Fig. 1C). Another important diagnostic feature of some sterile basidiomycetes is the production of spicules along the sides of hyphae (Fig. 1C, open arrow), with or without clamp connections (Fig. 1C, closed arrow), as in the case of *Schizophyllum commune* (Fig. 1C). Occasional dikaryons of this species may also produce basidiocarps (Fig. 1D). The recently reported species *Inonotus* (*Phellinus*) *tropicalis* (12, 15, 31), which is otherwise sterile in culture, may produce somewhat unusual hyphal elements known as setal hyphae (Fig. 1E); however, these types of hyphae may occur in other genera as well. Curved conidia, which are typical of *Hormographiella* species, may also be observed (Fig. 1F). The microscopic features of isolates included in this study are noted in Table 1. Finally, the ability of most basidiomycetes to grow on medium containing benomyl and their lack of growth on media containing cycloheximide further supported their probable identification.

A total of 168 filamentous isolates that had been identified as probable basidiomycetes by using the criteria cited above made up the study set that was sequenced.

Comparison of ITS and D1/D2 region BLAST results. Comparison of the top hits from the GenBank database for the ITS and D1/D2 regions showed a number of isolates that returned the same species name for both the ITS region and the D1/D2 region (Table 2). However, when the number of disagreements was considered for the two regions, comparative ITS-D1/D2 sequencing for this set of isolates showed an overall striking lack of agreement. Although the BLASTn results for each

TABLE 2. Comparison of GenBank top hits for the ITS and D1/D2 regions which agree[a]

Isolate	Organism ITS identified	% ITS identity	No. of ITS matches/ no. identified in GenBank	Organism D1/D2 identified	% D1/D2 identity	No. of D1/D2 matches/ no. identified in GenBank
06-4444	*Bjerkandera adusta*	99	698/705	*Bjerkandera adusta*	100	624/624
06-3787	*Bjerkandera adusta*	99	692/695	*Bjerkandera adusta*	100	583/583
05-1243	*Bjerkandera adusta*	100	583/583	*Bjerkandera adusta*	99	889/895
05-3095	*Bjerkandera adusta*	99	598/605	*Bjerkandera adusta*	100	624/624
05-1853	*Ceriporiopsis subvermispora*	97	751/774	*Ceriporiopsis subvermispora*	95	619/645
05-2504	*Fomitopsis feei*	99	646/647	*Fomitopsis feei*	99	646/647
06-3335	*Fomitopsis rosea*	98	638/647	*Fomitopsis rosea*	98	638/647
06-3906	*Irpex lacteus*	99	660/663	*Irpex lacteus*	100	560/560
07-312	*Mycorrhizal basidiomycete*	99	622/626	*Mycorrhizal basidiomycete*	100	646/646
05-597	*Oxyporus corticola*	100	605/605	*Oxyporus corticola*	99	647/648
05-1822	*Oxyporus corticola*	98	389/395	*Oxyporus corticola*	94	844/894
06-3281	*Peniophora cinerea*	96	693/716	*Peniophora cinerea*	98	590/602
06-3093	*Peniophora cinerea*	95	605/641	*Peniophora cinerea*	98	591/602
07-1076	*Peniophora cinerea*	95	505/541	*Peniophora cinerea*	98	591/602
06-2439	*Peniophora cinerea*	97	625/641	*Peniophora cinerea*	99	591/602
06-2581	*Peniophora cinerea*	96	617/641	*Peniophora cinerea*	97	591/602
06-2670	*Peniophora cinerea*	98	735/741	*Peniophora cinerea*	97	591/602
06-3035	*Peniophora cinerea*	91	775/841	*Peniophora cinerea*	97	587/602
05-1560	*Phlebia acerina*	92	756/785	*Phlebia acerina*	99	640/646
06-3159	*Phlebia radiata*	93	711/767	*Phlebia radiata*	99	646/647
06-3082	*Phlebia radiata*	88	629/766	*Phlebia radiata*	97	632/650
07-56	*Phlebia tremellosa*	97	646/664	*Phlebia tremellosa*	99	620/623
07-31	*Phlebia tremellosa*	99	620/623	*Phlebia tremellosa*	99	634/640
06-4410	*Phlebia tremellosa*	100	498/498	*Phlebia tremellosa*	100	603/603
06-4285	*Phlebia tremellosa*	98	644/651	*Phlebia tremellosa*	100	603/603
07-315	*Phlebia tremellosa*	98	585/592	*Phlebia tremellosa*	100	633/633
05-738	*Phlebia tremellosa*	98	585/592	*Phlebia tremellosa*	100	633/633
06-2422	*Phlebia tremellosa*	98	646/664	*Phlebia tremellosa*	99	620/623
06-2486	*Phlebia tremellosa*	96	646/664	*Phlebia tremellosa*	99	620/623
06-2644	*Phlebia tremellosa*	97	646/664	*Phlebia tremellosa*	93	575/623
06-3806	*Polyporus tricholoma*	98	647/655	*Polyporus tricholoma*	100	611/611
06-2860	*Psathyrella* cf. *gracilis*	98	694/706	*Psathyrella* cf. *gracilis*	99	644/646
06-3176	*Schizophyllum radiatum*	100	616/616	*Schizophyllum radiatum*	99	909/912
06-4124	*Termitomyces albuminosus*	99	638/639	*Termitomyces albuminosus*	99	622/623
05-1553	*Termitomyces albuminosus*	99	700/702	*Termitomyces albuminosus*	99	622/623
05-2641	*Termitomyces albuminosus*	99	700/702	*Termitomyces albuminosus*	99	622/623
06-3310	*Termitomyces albuminosus*	99	745/746	*Termitomyces albuminosus*	99	622/623
06-3259	*Termitomyces albuminosus*	99	745/746	*Termitomyces albuminosus*	99	622/623
06-3212	*Termitomyces albuminosus*	99	705/706	*Termitomyces albuminosus*	99	622/623
06-3194	*Termitomyces albuminosus*	99	732/733	*Termitomyces albuminosus*	99	621/623
06-3183	*Termitomyces albuminosus*	99	926/928	*Termitomyces albuminosus*	99	622/623
06-3080	*Termitomyces albuminosus*	99	705/706	*Termitomyces albuminosus*	99	622/623
05-3255	*Termitomyces albuminosus*	99	705/706	*Termitomyces albuminosus*	99	622/623
05-2677	*Termitomyces albuminosus*	99	745/746	*Termitomyces albuminosus*	99	622/623
06-2433	*Termitomyces albuminosus*	99	645/646	*Termitomyces albuminosus*	99	622/623
06-2571	*Termitomyces albuminosus*	99	745/746	*Termitomyces albuminosus*	98	622/623
06-2650	*Termitomyces albuminosus*	95	712/746	*Termitomyces albuminosus*	98	622/623
07-1074	*Termitomyces albuminosus*	98	745/746	*Termitomyces albuminosus*	97	615/623
05-2354	*Trametes versicolor*	99	759/763	*Trametes versicolor*	99	645–646

[a] Differences in sequence matches between multiple isolates of the same species and what was returned by BLAST reflect the different percent identities of multiple GenBank records for the same species, one of which had the closest identity to our sequence but which could differ with each search. The table was sorted alphabetically.

isolate yielded a basidiomycete identification to the species level for 99.4% (167/168) of the isolates, the inconsistency of the outputs for the two regions made it impossible to assign a conclusive identification for 70.8% (119/168) of the isolates (Table 3). At the least-stringent level, in which agreement between the two sequences needed to consist only of the same genus name, regardless of the percent identity (i.e., the ITS sequence identified *Phlebia tremellosa* with 95% identity; the D1/D2 sequence identified *Phlebia radiata* with 96% identity), only 48.8% (82/168) of the results were in agreement (Table 4). For genus and species agreement, regardless of the percent identity (i.e., the ITS sequence identified *Phlebia tremellosa* with 97% identity; the D1/D2 sequence identified *Phlebia tremellosa* with 93% identity), the results for only 28.6% (48/168) of the specimens agreed. For genus and species agreement with a cutoff of ≥97% identity, the results for only 21.4% (36/168) of the specimens agreed. Further analysis showed that of the 168 sequences, the sequence of only a single isolate (0.6%) displayed matching ITS region- and D1/D2 region-based genus and species names with 100% identity.

Comparison of ITS and D1/D2 GenBank deposits. In order to investigate possible causes for the low frequency of agreement

TABLE 3. Comparison of GenBank top hits for the ITS and D1/D2 regions which disagree[a]

Isolate	Organism ITS identified	% ITS identity	No. of ITS matches/ no. identified in GenBank	Organism D1/D2 identified	% D1/D2 identity	No. of D1/D2 matches/ no. identified in GenBank
07-551	*Antrodia albida*	99	443/446	*Fomes fomentarius*	98	636/646
06-3321	*Antrodia malicola*	96	348/360	*Bjerkandera adusta*	95	1136/1194
06-2304	*Antrodia malicola*	96	448/460	*Bjerhandera adusta*	95	736/794
06-2544	*Antrodia malicola*	96	448/460	*Bjerhandera adusta*	97	745/794
06-4454	*Bjerkandera adusta*	98	694/706	*Antrodia malicola*	99	644/646
06-4450	*Bjerkandera adusta*	96	604/627	*Antrodia malicola*	100	644/644
06-4161	*Bjerkandera adusta*	98	675/685	*Antrodia malicola*	100	646/646
06-3795	*Bjerkandera adusta*	100	583/583	*Thanatephorus cucumeris*	99	591/596
06-3769	*Bjerkandera adusta*	92	630/668	*Oudemansiella canarii*	92	630/670
06-2441	*Bjerkandera adusta*	98	894/906	*Antrodia malicola*	99	644/646
06-2552	*Bjerkandera adusta*	98	690/706	*Antrodia malicola*	99	644/646
06-2725	*Bjerkandera adusta*	97	690/706	*Antrodia malicola*	99	644/646
06-2683	*Bjerkandera adusta*	100	583/583	*Thanatephorus cucumeris*	98	591/596
06-3002	*Bjerkandera adusta*	97	604/627	*Antrodia malicola*	100	644/644
06-3001	*Bjerkandera adusta*	100	583/583	*Thanatephorus cucumeris*	99	591/596
06-2939	*Bjerkandera adusta*	96	604/627	*Antrodia malicola*	100	644/644
05-2954	*Coprinopsis cinera*	100	626/626	*Coprinopsis domesticus*	99	581/583
07-865	*Coprinopsis cinera*	100	626/626	*Coprinopsis domesticus*	99	581/583
05-567	*Coprinopsis cinerea*	100	639/639	*Coprinus trisporus*	99	613/614
06-3970	*Coprinus echinosporus*	93	473/504	*Coprinus trisporus*	100	612/612
06-2354	*Coprinus echinosporus*	97	473/504	*Coprinus trisporus*	100	612/612
06-2687	*Coprinus echinosporus*	95	473/504	*Coprinus trisporus*	100	612/612
06-2949	*Coprinus echinosporus*	93	473/504	*Coprinus trisporus*	100	612/612
06-3497	*Coprinus radians*	100	658/658	*Phlebia chrysocreas*	95	582/608
06-3341	*Coprinus radians*	100	658/658	*Phlebia chrysocreas*	95	582/608
05-1063	*Coprinus radians*	100	637/637	*Ceriporiopsis subvermispora*	99	622/624
05-1575	*Coriolopsis caperata*	96	651/673	*Microporus affinis*	98	638/648
05-459	*Fomes fomentarius*	97	751/774	*Microporus affinis*	98	636/646
06-2401	*Fomes fomentarius*	97	751/774	*Microporus affinis*	98	636/646
06-2563	*Fomes fomentarius*	96	751/774	*Microporus affinis*	98	636/646
06-3768	*Fomitopsis pinicola*	95	736/794	*Fomitopsis feei*	99	589/590
07-495	*Hymenochaete spreta*	98	699/707	*Hydnochaete olivacea*	99	654/657
06-3994	*Irpex lacteus*	99	660/663	*Phlebia tremellosa*	98	630/644
06-3888	*Irpex lacteus*	99	622/625	*Antrodia malicola*	99	633/635
06-3788	*Irpex lacteus*	93	450/468	*Trametes maxima*	93	450/468
06-3536	*Oudemansiella canarii*	97	429/437	*Polyporus brumalis*	100	620/620
07-1092	*Oudemansiella canarii*	97	329/337	*Polyporus brumalis*	100	620/620
06-2341	*Oudemansiella canarii*	98	431/437	*Polyporus brumalis*	100	620/620
06-2721	*Oudemansiella canarii*	97	329/337	*Polyporus brumalis*	100	620/620
05-2369	*Oxyporus corticola*	93	819/874	*Panus strigellus*	97	751/775
06-2629	*Peniophora cinerea*	93	701/779	*Phlebia tremellosa*	98	591/596
06-3869	*Phanerochaete carnosa*	93	450/468	*Phanerochaete velutina*	92	557/603
06-3499	*Phanerochaete velutina*	98	636/646	*Phanerochaete sordida*	98	735/747
05-2219	*Phlebia acerina*	93	719/776	*Phlebia radiata*	96	625/647
05-2777	*Phlebia acerina*	96	503/526	*Phlebia radiata*	95	620/648
05-2582	*Phlebia acerina*	96	503/526	*Phlebia radiata*	95	620/648
05-2742	*Phlebia lilascens*	94	585/616	*Coprinellus disseminatus*	100	621/621
05-3058	*Phlebia lilascens*	99	667/673	*Coprinopsis domesticus*	100	621/621
05-2353	*Phlebia radiata*	92	770/829	*Schizophyllum radiatum*	96	626/646
05-2587	*Phlebia radiata*	94	844/894	*Hymenochaete spreta*	95	850/894
07-797	*Phlebia radiata*	93	711/767	*Phlebia uda*	99	646/647
07-864	*Phlebia radiata*	93	511/567	*Phlebia uda*	99	646/647
06-2723	*Phlebia radiata*	93	711/767	*Polyporus brumalis*	99	646/647
06-2997	*Phlebia radiata*	93	811/867	*Polyporus brumalis*	99	646/647
06-3460	*Phlebia subserialis*	99	578/579	*Phlebia chrysocreas*	95	582/608
05-2308	*Phlebia subserialis*	99	554/556	*Phlebia chrysocreas*	95	619/645
05-2474	*Phlebia subserialis*	99	563/564	*Phlebia chrysocreas*	95	619/645
06-3223	*Phlebia uda*	93	662/702	*Schizophyllum commune*	100	613/613
05-2738	*Phlebia uda*	93	662/702	*Phlebia subochracea*	94	699/751
07-729	*Phlebia uda*	93	662/702	*Termitomyces albuminosus*	99	622/623
07-1095	*Phlebia uda*	93	662/702	*Phanerochaete velutina*	99	635/636
06-2358	*Phlebia uda*	93	662/702	*Bjerkandera adusta*	99	642/646
06-2624	*Phlebia uda*	93	662/702	*Coprinus quadrifidus*	98	636/647
06-4057	*Polyporus brumalis*	98	633/640	*Termitomyces albuminosus*	99	622/623
06-3349	*Polyporus brumalis*	97	627/651	*Lentinus bertieri*	99	611/612
05-2588	*Polyporus brumalis*	97	647/664	*Lentinus bertieri*	99	611/613

Continued on following page

TABLE 3—*Continued*

Isolate	Organism ITS identified	% ITS identity	No. of ITS matches/ no. identified in GenBank	Organism D1/D2 identified	% D1/D2 identity	No. of D1/D2 matches/ no. identified in GenBank
05-1932	*Rhizochaete filamentosa*	93	617/679	*Phlebiopsis gigantea*	96	625/648
05-2586	*Rhizochaete filamentosa*	94	693/711	*Phlebiopsis gigantea*	96	628/650
06-3298	*Rhizochaete filamentosa*	93	592/652	*Phlebiopsis gigantea*	96	628/650
06-3297	*Rhizochaete filamentosa*	92	677/749	*Phanerochaete velutina*	99	639/645
06-3094	*Rhizochaete filamentosa*	93	692/752	*Phlebiopsis gigantea*	96	628/650
05-2061	*Rhizochaete fouquieriae*	95	701/779	*Trametes versicolor*	96	625/648
05-2164	*Rhizochaete fouquieriae*	98	389/395	*Panus strigellus*	99	611/612
05-1416	*Schizophyllum commune*	94	644/694	*Hymenochaete spreta*	96	850/894
05-2239	*Schizophyllum commune*	99	634/637	*Schizophyllum radiatum*	100	613/613
05-1442	*Schizophyllum commune*	93	917/979	*Polyporus brumalis*	97	751/774
06-2442	*Schizophyllum commune*	94	544/594	*Hymenochaete spreta*	96	850/894
06-2432	*Schizophyllum commune*	95	501/579	*Trametes versicolor*	96	625/648
06-2420	*Schizophyllum commune*	94	644/694	*Hymenochaete spreta*	96	850/894
06-2729	*Schizophyllum commune*	95	701/779	*Trametes versicolor*	97	625/648
06-2641	*Schizophyllum commune*	95	731/779	*Trametes versicolor*	96	625/648
06-2807	*Schizophyllum commune*	94	644/694	*Hymenochaete spreta*	96	850/894
06-3190	*Schizophyllum radiatum*	99	625/626	*Termitomyces albuminosus*	99	625/626
06-3182	*Schizophyllum radiatum*	92	892/960	*Schizophyllum commune*	91	711/899
07-1061	*Schizophyllum radiatum*	99	625/626	*Termitomyces albuminosus*	99	625/626
06-2544	*Schizophyllum radiatum*	99	625/626	*Termitomyces albuminosus*	99	625/626
07-1060	*Schizophyllum radiatum*	99	625/626	*Termitomyces albuminosus*	99	625/626
06-4137	*Termitomyces albuminosus*	99	609/610	*Phlebia tremellosa*	99	634/640
06-3466	*Termitomyces albuminosus*	99	700/702	*Phanerochaete sordida*	98	735/747
05-1037	*Termitomyces albuminosus*	97	751/774	*Ceriporiopsis subvermispora*	98	616/623
05-2112	*Termitomyces albuminosus*	99	609/614	*Trametes versicolor*	99	642/645
05-2269	*Termitomyces albuminosus*	96	765/789	*Ceriporiopsis subvermispora*	99	616/623
06-2736	*Termitomyces albuminosus*	99	600/602	*Phanerochaete sordida*	97	735/747
06-3057	*Termitomyces albuminosus*	99	700/702	*Phanerochaete sordida*	98	735/747
06-3924	*Thanatephorus cucumeris*	99	624/626	*Antrodia malicola*	99	644/646
06-3821	*Thanatephorus cucumeris*	99	624/626	*Bjerkandera adusta*	100	583/583
06-4341	*Trametes maxima*	96	781/813	*Donkioporia expansa*	97	628/645
06-4103	*Trametes maxima*	95	638/682	*Polyporus brumalis*	100	620/620
06-3621	*Trametes maxima*	94	517/575	*Polyporus brumalis*	100	620/620
06-3308	*Trametes maxima*	94	635/650	*Polyporus tricholoma*	97	621/622
05-3281	*Trametes maxima*	95	638/682	*Polyporus brumalis*	100	620/620
07-866	*Trametes maxima*	95	638/682	*Polyporus brumalis*	100	620/620
06-2734	*Trametes maxima*	95	638/682	*Polyporus brumalis*	100	620/620
06-2951	*Trametes maxima*	95	781/813	*Donkioporia expansa*	98	628/645
06-2947	*Trametes maxima*	95	838/882	*Polyporus brumalis*	100	620/620
06-2833	*Trametes maxima*	96	781/813	*Donkioporia expansa*	97	628/645
05-2034	*Trametes ochracea*	97	615/636	*Trametes hirsuta*	99	644/646
05-2341	*Trametes ochracea*	97	751/774	*Trametes versicolor*	96	625/648
06-3320	*Trametes versicolor*	94	554/569	*Bjerkandera adusta*	100	583/583
05-679	*Trametes versicolor*	94	844/894	*Trametes lactinea*	100	613/613
05-3313	*Trametes versicolor*	93	750/798	*Trametes lactinea*	100	613/613
05-3368	*Trametes versicolor*	94	630/681	*Trametes lactinea*	100	611/611
05-2585	*Trametes versicolor*	93	750/798	*Trametes lactinea*	100	613/613
07-793	*Trametes versicolor*	93	750/798	*Trametes lactinea*	100	613/613
05-2661	*Trametes versicolor*	93	750/798	*Trametes lactinea*	100	613/613
06-2362	*Trametes versicolor*	93	750/798	*Trametes lactinea*	100	613/613
06-2536	*Trametes versicolor*	93	750/798	*Trametes lactinea*	100	613/613
06-2685	*Trametes versicolor*	93	750/798	*Trametes lactinea*	100	613/613
06-2839	*Trametes versicolor*	93	750/798	*Trametes lactinea*	100	613/613

[a] Differences in sequence matches between multiple isolates of the same species and what was returned by BLAST reflect the different percent identities of multiple GenBank records for the same species, one of which had the closest identity to our sequence but which could differ with each search. The table was sorted alphabetically on the basis of the ITS name.

for the ITS and D1/D2 regions, the GenBank database was searched for the presence of sequence deposits that corresponded to these two sequences for each species. This analysis revealed that there were no entries in GenBank for 14% of the top ITS hits (7/50) and 16% (8/50) of the top D1/D2 hits for the isolates on our list (Table 5). Therefore, 30% of the species that were identified in this study had either an ITS or a D1/D2 sequence that matched a deposit in GenBank, but not both.

Analysis of ITS and D1/D2 GenBank deposit sequence lengths. The top hits for each BLAST query for both the ITS and the D1/D2 regions with an identity of 97% or greater were evaluated for their completeness, which was defined as a se-

TABLE 4. ITS-D1/D2 BLAST output comparison

% Identity cutoff	No.[a]	% Agreement
Genus only agreement[b]	82	48.8
Genus + species agreement,[c] any % identity	48	28.6
Genus + species agreement, one ≥97% identity	48	28.6
Genus + species agreement, both ≥97% identity	36	21.4
Genus + species agreement, one ≥98% identity	43	25.6
Genus + species agreement, both ≥98% identity	32	19
Genus + species agreement, one ≥99% identity	36	21.4
Genus + species agreement, both ≥99% identity	24	14.3
Genus + species agreement, one 100% identity	12	7.1
Genus + species agreement, both 100% identity	1	0.6

[a] Number of isolates of 168 isolates tested with given identity.
[b] Any percent identity.
[c] Genus plus species agreement represents BLAST outputs in which the ITS genus and species name matched the D1/D2 genus and species name.

quence whose length matched the length of our query sequence, excluding internal deletions or insertions. Comparison of GenBank deposit sequence lengths of both the ITS and the D1/D2 regions for each isolate showed that the deposit entries for each region were largely truncated compared to the lengths of the regions that we sequenced. Of the 50 species represented, only 8% of the ITS regions and 10% of the D1/D2 regions had complete sequence data in GenBank (Table 5).

Since most of the species that we identified are rare and in many cases had only a single GenBank deposit, we selected six species that were the most redundant from the BLAST output list (Table 2) and obtained the sequence lengths from each record with the highest percent identity. Sequences were recovered for *Bjerkandera adusta* (GenBank accession number EU918694), *Coprinellus disseminatus* (GenBank accession number FN386275), *Fomitopsis rosea* (GenBank accession number DQ491412), *Irpex lacteus* (GenBank accession number FJ462768.1), *Phanerochaete sordida* (GenBank accession number EU118653.1), and *Trametes versicolor* (GenBank accession number FJ810146). The ITS sequence analysis (Fig. 2A) showed that regardless of the species, there were no complete sequences compared to our ITS sequences. The most complete sequence of *Coprinellus disseminatus* found in GenBank was 697 bp (GenBank accession number FN386275), whereas the ITS sequence that we obtained was 702 bp. The other GenBank ITS sequences varied in length and were found to be incomplete as well. The ITS sequence lengths obtained from GenBank ranged from 95% to 99% compared to the complete ITS sequences that we derived by sequencing with primers ITS-1 and ITS-4. The D1/D2 sequence length data (Fig. 2B) also proved to be largely incomplete. The most complete sequence of *Bjerkandera adusta* found in the GenBank database was 654 bp (GenBank accession number AB096738), whereas the D1/D2 sequence that we obtained was 660 bp. The D1/D2 sequence lengths obtained from GenBank ranged from 41% to 99% complete compared to the complete D1/D2 sequences that we obtained by sequencing with primers NL-1 and NL-4. These data indicate that many of the current GenBank sequences for basidiomycetes have incomplete sequence data for the regions that we used for identification. Importantly, these results were obtained for the most redundant species recovered from our BLAST searches and therefore would be expected to have a higher likelihood for a complete deposit due to the presence of multiple records.

TABLE 5. Presence of species-specific GenBank ITS and D1/D2 deposits

Species	GenBank record[a]	
	ITS region	D1/D2 region
Antrodia albida	X (complete)	X (partial)
Antrodia malicola	X (complete)	X (partial)
Bjerkandera adusta	X (partial)	X (partial)
Ceriporiopsis subvermispora	X (partial)	X (partial)
Coprinellus disseminatus	X (partial)	X (partial)
Coprinopsis cinera	X (partial)	No deposit
Coprinopsis domesticus	No deposit	X (partial)
Coprinus echinosporus	X (partial)	No deposit
Coprinus quadrifidus	X (partial)	X (partial)
Corpinus radians	X (partial)	X (partial)
Corpinus trisporus	No deposit	X (partial)
Coriolopsis caperata	X (partial)	X (partial)
Donkiopora expansa	X (partial)	X (partial)
Fomes fomentarius	X (partial)	X (partial)
Fomitopsis feei	X (partial)	X (partial)
Fomitopsis pinicola	X (partial)	X (partial)
Fomitopsis rosea	X (complete)	X (partial)
Hydnochaete olivacea	X (partial)	No deposit
Hymenochaete spreta	X (partial)	X (partial)
Irpex lacteus	X (partial)	X (partial)
Lentinus bertieri	No deposit	X (partial)
Microporus affinis	No deposit	X (partial)
Oudemansiella canarii	X (partial)	No deposit
Oxyporus corticola	X (partial)	X (partial)
Panus strigellus	No deposit	X (partial)
Peniophora cinerea	X (partial)	X (partial)
Phanerochaete carnosa	X (partial)	No deposit
Phanerochaete sordida	X (partial)	X (partial)
Phanerochaete velutina	X (partial)	X (partial)
Phlebia acerina	X (partial)	X (partial)
Phlebia chrysocreas	No deposit	X (complete)
Phlebia lilascens	No deposit	X (partial)
Phlebia radiata	X (partial)	X (partial)
Phlebia subochracea	X (partial)	X (partial)
Phlebia subserialis	X (partial)	X (partial)
Phlebia tremellosa	X (partial)	X (partial)
Phlebia uda	X (partial)	X (complete)
Phlebiopsis gigantea	X (partial)	X (partial)
Polyporus brumalis	X (partial)	X (complete)
Polyporus tricholoma	X (partial)	X (partial)
Rhizochaete filamentosa	X (partial)	X (complete)
Rhizochaete fouquieriae	X (partial)	No deposit
Schizophyllum commune	X (partial)	X (partial)
Schizophyllum radiatum	X (partial)	X (complete)
Termitomyces albuminosus	X (complete)	No deposit
Thanatephorus cucumeris	X (partial)	No deposit
Trametes lactinea	X (partial)	X (partial)
Trametes maxima	X (partial)	X (partial)
Trametes ochracea	X (partial)	X (partial)
Trametes versicolor	X (partial)	X (partial)

[a] X, a sequence deposit was made for this species. No deposit, no GenBank record could be found for the corresponding sequence; partial and complete, the sequence length between the ITS-1 and ITS-4 primers (ITS region) or the NL-1 and NL-4 primers (D1/D2 region).

DISCUSSION

The frequency of human mycoses due to filamentous fungi is steadily increasing, and mycoses mostly affect defined risk groups, such as immunocompromised or severely ill pa-

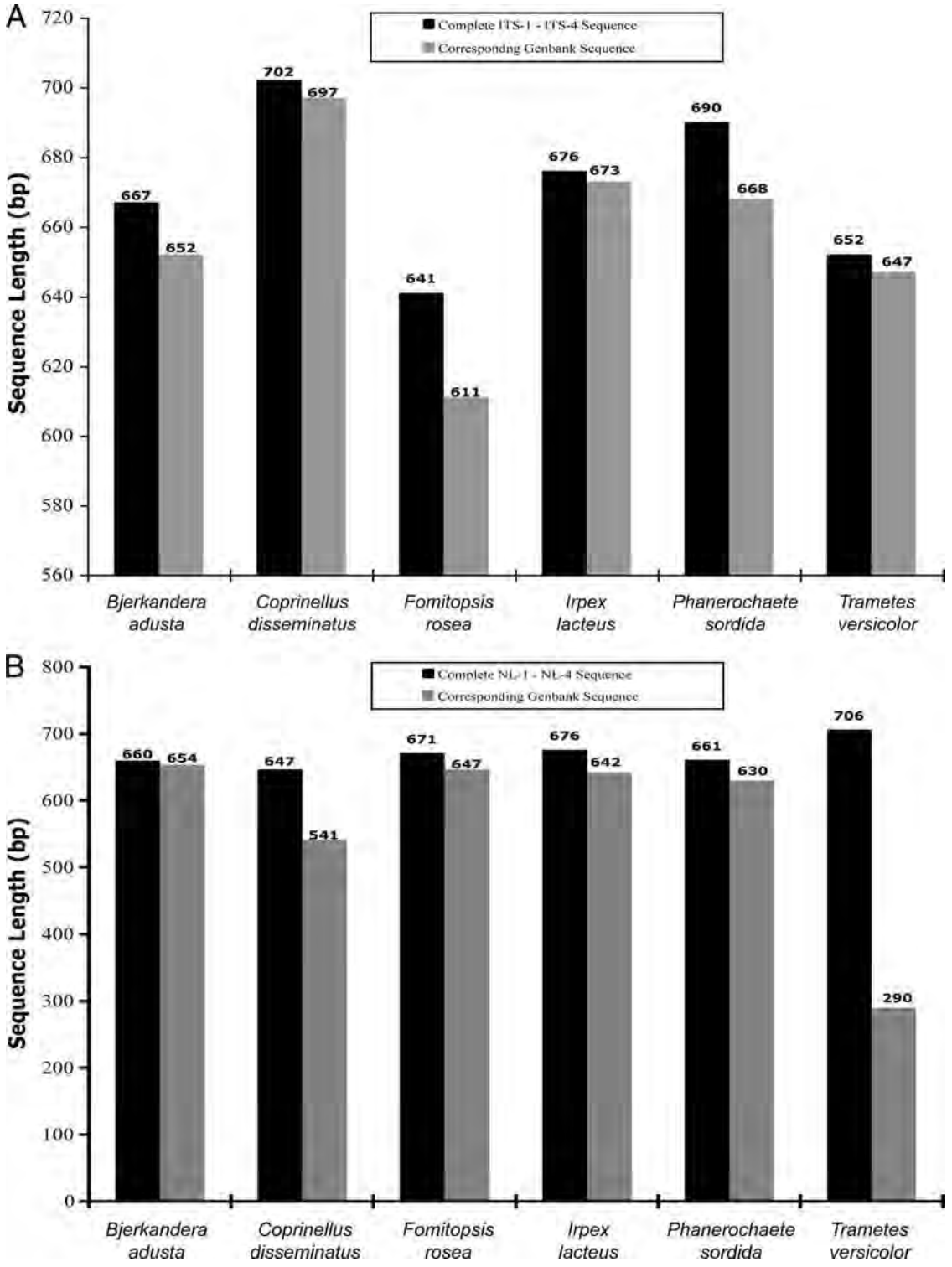

FIG. 2. Comparison of ITS and D1/D2 sequence lengths. (A) Comparison of ITS lengths to ITS lengths in GenBank. The sequence lengths of the ITS regions of our isolates were compared to those found in GenBank. The six species represented here were chosen on the basis of being the most redundant among the results from the BLASTn search. (B) Comparison of D1/D2 lengths to D1/D2 lengths in GenBank. The sequence lengths of the D1/D2 regions of our isolates were compared to those found in GenBank.

tients. In addition to the well-known opportunistic basidiomycetous pathogen *Cryptococcus neoformans*, other basidiomycetous yeasts such as *Malassezia* spp. and *Rhodotorula* spp. are now considered emergent opportunistic pathogens and are recovered at increasing frequencies (6, 14, 20, 22). Basidiomycetous molds, with few exceptions, are rarely recovered as human pathogens because of the difficulty identifying these fungi or the difficulty distinguishing colonizers

from invasive isolates in patient specimens. Sterile and/or arthroconidium-forming basidiomycetes are a subset of this class and cannot be conclusively identified by standard phenotypic methods because they do not produce distinguishing structures. Although these mostly sterile isolates may be morphologically identified as basidiomycetes when clamp connections are present, many genera of basidiomycetes do not produce clamp connections in culture. Consequently, they may simply be described as nonsporulating molds with unknown clinical significance.

In a study by Pounder et al., which also used a sequencing strategy for identification, 31 of the 48 (65%) isolates were classified as basidiomycetes (23) by use of a sequence derived from the ITS region. Under the cutoff criteria of a sequence length of at least 400 bp, ≥99% identity for a species-level identification, and ≥93% identity for a genus-level identification, 92% of the isolates were identified to the genus level and 79% were identified to the species level. Because of the relatively high identification rate, we decided to use a similar strategy to identify our isolates. A large number of the initial ITS sequences that we obtained did not meet the 97% cutoff criterion that we established for identity. Therefore, we decided to add the D1/D2 region as a second locus, under the assumption that the results from D1/D2 searches would yield more identities higher than 97%, thereby allowing an identification. However, we were surprised to find that while in many cases we obtained a D1/D2 identity of ≥97%, we observed a striking amount of disagreement between the best hit (the highest level of identity) for the ITS search and the best hit for the D1/D2 search. The agreement between the two sequences for the same isolate was only 28.6% at any level of identity, whereas with a more stringent cutoff of ≥97% identity, agreement occurred only 21.4% of the time. We suspect that this low level of agreement would likely be the same for any mold that is rarely studied at the molecular level, whether it is sterile or not, due to the absence of searchable data in GenBank.

The low level of ITS-D1/D2 agreement led us to investigate why the results were so disparate. Of the 50 species that we identified, almost a third did not have a GenBank deposit for the ITS region or the D1/D2 region. When all significant hits (≥97% identity) were considered for each search output, two-thirds (66%) of the records had either an ITS deposit or a D1/D2 deposit, but not both. As a result of this discrepancy, error can be introduced during the BLAST search output when the next-highest identity, which will be a different species, becomes the top hit in the search. We also found that the sequences in GenBank were largely incomplete compared to our query sequences. It is not clear how much sequence would need to be truncated from either or both ends before a significant impact on identity occurs; however, sequence alignments demonstrate that sequence variation can occur very close (within a few bases) to the primer sites that we used (data not shown). These variable regions may not be present in the sequence if the sequence is truncated due to single-stranded sequencing, if the sequence is derived from a different primer combination or a partially overlapping region, or if the sequencing run terminates and does not proceed through the primers. These observations, combined with known GenBank issues such as nomenclature errors (5) and poor-quality deposits (18, 23), can complicate sequence-based identification. In fact, fungal GenBank deposits may be more adversely affected by issues involving nomenclature than GenBank deposits for other microbial organisms. Few investigators working with fungi outside of classical mycology are well versed in the rules governing how and when the anamorph and teleomorph nomenclature is properly used. Similarly, isolates may be identified by their obsolete or synonymous names, and selection of the currently accepted name is difficult even for classical mycologists, since names are often changed on the basis of basic research, including some of the molecular techniques used in this study, and may not be widely reported or even accepted. The sequences of basidiomycetes in the GenBank database, with the possible exception of those of *C. neoformans*, may be more adversely affected by these issues, since taxonomic studies of basidiomycete species pathogenic for humans may be lagging similar studies of other fungi, such as the aspergilli or the fusaria, for which detailed analysis has resulted in revised classifications (2, 3). These issues were not addressed in the data analysis, since the study focused on the actual GenBank outputs; consequently, it is possible that the levels of agreement would improve slightly due to a correct agreement being masked by the erroneous or inconsistent naming of the deposit.

Our results and the results of Pounder et al. (23) suggest that sterile molds recovered from human clinical specimens may comprise a substantial number of basidiomycetes. In fact, our study utilized a subset of sterile and/or arthroconidium-producing isolates from human clinical specimens phenotypically identified as probable basidiomycetes (on the basis of the morphological criteria that we used for our study) that had been sent to the Fungus Testing Laboratory. Both studies had six species in common, including *Polyporus tricholoma*, *Irpex lacteus*, *Schizophyllum commune*, *Phlebia subserialis*, *Trametes versicolor*, and *Thanatephorus cucumeris*. While it is highly likely that most filamentous basidiomycetes identified from clinical specimens are clinically insignificant because they are noncolonizers abundant in ambient air, a number of our specimens were from sites other than the respiratory tract that are normally sterile (i.e., cerebrospinal fluid). The host status, the route of infection, and the shear number and variety of fungal elements that a patient is exposed to likely determine whether a basidiomycosis can occur.

While this study has highlighted issues that need to be carefully considered when sequence-based identification is employed, sequence-based identification has some major diagnostic strengths and continues to be extremely useful to our group for fungal identification. It clearly has great diagnostic value for common fungi and/or fungi that have numerous GenBank deposits. The sequence data in GenBank are also useful if they are combined with additional nonsequence data, even if the sequencing results are somewhat ambiguous. In fact, our sequencing results for the 168 isolates were in complete agreement with the preliminary morphological results, in that all BLAST results were consistent with the organism being a basidiomycete. However, as a general rule, on the basis of the results of this study, we now utilize both the ITS and D1/D2 regions when we make sequence-based identifications for any fungus and double check if there is disagreement to make sure there is a GenBank deposit for both sequences. While this strategy does not guarantee that the sequence results will be 100% accurate, it can rapidly reveal whether there are enough

data in GenBank for sequencing to even be used in the identification process. In our specific study, unfortunately, there does not seem to be enough data in GenBank to identify any unknown sterile basidiomycete with a high degree of confidence by ITS and/or D1/D2 sequencing.

As sequencing moves toward broader acceptance in the clinical laboratory, an important challenge to be overcome will be the development of a process that can provide a platform that certification bodies (the Clinical Laboratory Improvement Amendments [CLIA], the College of American Pathologists [CAP]) can standardize. In fact, guidelines are now being established to facilitate standardization (11). Evaluation of the data source should clearly be included in this platform, a major part of which should be a determination of how databases, whether they are public, such as GenBank, or private, could fit into the process. Unfortunately, the choice of database is not going to be a trivial issue. Despite the known errors with GenBank records, the depth of the sequences with regard to the number of potential species included in the database cannot be matched. Even imprecise GenBank records can be informative in some cases, since some taxonomic information may be identifiable, despite incorrect genus or species names. Conversely, private or closed databases may be more accurate due to the confirmation of each entry and the deposit of high-quality sequences. However, these databases will likely sacrifice species diversity and redundancy due to the smaller number of entries. Despite this shortcoming, a closed database may be more amenable to standardization, particularly if sequences are generated specifically for the database (versus downloading from another source), since primers, completeness, and identities can be standardized and confirmed.

In summary, this study has shown that in addition to the well-known concerns with the use of the sequences in a public database for sequence-based identification, missing data can also contribute to erroneous conclusions during searches. These errors may be caught for fungi for which substantial phenotypic data are available for comparison to the sequencing results; however, when there are few phenotypic data, such as for sterile basidiomycetes or other molds, erroneous conclusions could be quite common.

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Oral and Parenteral Therapeutic Options for Outpatient Urinary Infections Caused by *Enterobacteriaceae* Producing CTX-M Extended-Spectrum β-Lactamases▽

Vidhya Prakash,[1] James S. Lewis II,[2] Monica L. Herrera,[3] Brian L. Wickes,[3] and James H. Jorgensen[2,3,4]*

San Antonio Military Medical Center, Infectious Disease Service, San Antonio, Texas 78234[1]; Department of Medicine, The University of Texas Health Science Center and Pharmacy Service, University Hospital, San Antonio, Texas 78229[2]; Department of Microbiology and Immunology, The University of Texas Health Science Center, San Antonio, Texas 78229[3]; and Department of Pathology, The University of Texas Health Science Center, San Antonio, Texas 78229[4]

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Effective therapeutic options are needed for community-onset urinary tract infections due to *Escherichia coli* strains that produce CTX-M extended-spectrum β-lactamases. We examined 46 urinary isolates producing CTX-M against several oral or long-acting parenteral antimicrobial agents. Approximately 90% were susceptible to fosfomycin and to a combination of cefdinir plus amoxicillin-clavulanate. All were susceptible to ertapenem.

Since the early 1990s, *Escherichia coli* isolates that produce CTX-M extended-spectrum β-lactamases (ESBLs) have emerged as a serious cause of urinary tract infections (UTIs) in the community (18). Mortality in the more severe infections, particularly those progressing to bacteremia, is as high as 60.8% (14). Chances of survival increase with appropriate initial antibiotic coverage, while delay in proper therapy is associated with increased mortality (14). Empirical antibiotic therapy, particularly in the outpatient setting, is problematic as most of these organisms are resistant to fluoroquinolones, trimethoprim-sulfamethoxazole, oral cephalosporins, and amoxicillin-clavulanate (18, 22). The primary goal of this investigation was to identify potential treatment options for outpatient UTIs with these organisms. We tested several candidate oral antibiotics and one long-acting parenteral agent against a collection of genetically characterized ESBL-producing isolates.

The ESBLs produced by each isolate were characterized by PCR amplification followed by sequencing of PCR products as previously described (13). A total of 45 UTI isolates (predominantly *E. coli*) that produced a CTX-M alone (40 producing CTX-M15, three producing CTX-M16, and one each producing CTX-M8 and CTX-M14) and one isolate that produced a CTX-M15 and an SHV-2 ESBL were examined along with 11 isolates that produced only SHV (four producing SHV-12, three producing SHV-2, and three producing SHV-5) or TEM-10 ESBLs. All isolates were recovered between 2002 and 2008. Isolates were stored frozen at −70°C in skim milk and subcultured twice prior to susceptibility testing. Each isolate was tested for susceptibility to fosfomycin by the CLSI agar dilution method (4) and to ciprofloxacin, doxycycline, ertapenem, and nitrofurantoin and to a novel combination of cefdinir plus a fixed concentration of amoxicillin-clavulanate by the CLSI broth microdilution method (4). For testing the unique combination of cefdinir and amoxicillin-clavulanate, the cefdinir was diluted in the usual twofold dilution scheme in a fixed concentration of 8 μg/ml amoxicillin and 4 μg/ml clavulanate. Both cefdinir and amoxicillin-clavulanate were tested separately in the normal twofold dilution format to ascertain their activities when tested alone. The calculation of the percentage of isolates susceptible to the three-drug combination was based upon the cefdinir component and use of the approved cefdinir-susceptible breakpoint of ≤1 μg/ml (5).

Results are summarized in Table 1. Approximately 90% of urinary CTX-M ESBL-producing isolates were susceptible to the combination of cefdinir plus amoxicillin-clavulanate and to fosfomycin. One hundred percent of isolates were susceptible to ertapenem. Nitrofurantoin was active against 73.9% of isolates, while only 10.9% and 4.3% were susceptible to doxycycline and ciprofloxacin, respectively. Testing of the 11 SHV or TEM ESBL-producing strains showed similar results, with the exception of nitrofurantoin, to which a majority were resistant (Table 2).

E. coli strains that produce CTX-M ESBLs, primarily found in community sources, are becoming widely prevalent worldwide, most notably in Europe and Canada (16, 17, 18). The emergence of community-onset UTIs in particular is concerning as they are mostly resistant to oral antibiotics (3, 7, 8, 13, 22, 23, 25). One study from Spain reported a threefold rise in community-onset UTIs caused by ESBL-producing *E. coli* over a 3-year period, most of which were also resistant to trimethoprim-sulfamethoxazole and fluoroquinolones (3). Another study from the United Kingdom revealed a similar trend in which 24% of 291 CTX-M-producing *E. coli* isolates (mostly urinary in origin) came from the community, most of them also being resistant to fluoroquinolones, trimethoprim-sulfamethoxazole, and tetracycline (25). Resistance to commonly prescribed oral antibiotics leads to inadequate empirical therapy and potentially the development of more severe infections including bacteremia. One study showed that with ESBL-producing *E. coli* strains isolated from nonhospitalized patients

* Corresponding author. Mailing address: Department of Pathology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900. Phone: (210) 567-4088. Fax: (210) 567-2367. E-mail: jorgensen@uthscsa.edu.

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TABLE 1. MIC_{50}s, MIC_{90}s, and percent susceptibilities of urine CTX-M ESBL-producing isolates to the study antimicrobial agents

Drug	Value for CTX-M ESBL-producing isolates (n = 46)		
	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	% Susceptible
Fosfomycin	0.5	64	91.3
Nitrofurantoin	16	64	73.9
Doxycycline	16	16	10.9
Ciprofloxacin	32	32	4.3
Cefdinir alone	16	16	0
Amoxicillin-clavulanate alone	32/4	32/4	10.9
Cefdinir plus amoxicillin-clavulanate	0.25[a]	2[a]	89.1
Ertapenem	0.06	0.25	100

[a] Cefdinir MIC in the presence of a fixed concentration of 8 μg/ml amoxicillin and 4 μg/ml clavulanate; percent-susceptible value is based upon the approved cefdinir-susceptible breakpoint of <1 μg/ml (5).

TABLE 2. MIC_{50}s, MIC_{90}s, and percent susceptibilities of urine SHV or TEM ESBL-producing isolates

Drug	Value for SHV or TEM ESBL-producing isolates (n = 11)		
	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	% Susceptible
Fosfomycin	4	8	100
Nitrofurantoin	64	64	45.5
Doxycycline	16	16	18.2
Ciprofloxacin	32	32	36.4
Cefdinir alone	4	16	0
Amoxicillin-clavulanate alone	32/4	32/4	45.5
Cefdinir plus amoxicillin-clavulanate	0.06[a]	8[a]	81.8
Ertapenem	0.12	1	100

[a] Cefdinir MIC in the presence of a fixed concentration of 8 μg/ml amoxicillin and 4 μg/ml clavulanate; percent-susceptible value is based upon the approved cefdinir-susceptible breakpoint of <1 μg/ml (5).

with UTIs, 5 out of 37 patients became bacteremic, requiring hospitalization due to treatment with inadequate initial empirical therapy (22). Another study reported a 4.1% prevalence of community-onset bacteremia caused by ESBL-producing *E. coli* with an associated mortality rate of 21.1% (9).

The rise in community-onset UTIs with ESBL-producing *E. coli* strains raises the question of how to treat these infections effectively on an outpatient basis. A recent case control study reported a 93% cure rate for cystitis using amoxicillin-clavulanate for community-onset UTIs with ESBL-producing *E. coli* strains that were susceptible to that combination. However, 29% of the isolates were resistant to amoxicillin-clavulanate (21). Our study revealed that, while no isolates were susceptible to cefdinir alone and only 10.9% of isolates were susceptible to amoxicillin-clavulanate alone, the addition of a fixed concentration of amoxicillin-clavulanate to cefdinir raised the percentage of isolates susceptible to 89.1% based upon a MIC of ≤1 μg/ml of cefdinir in the presence of the β-lactamase inhibitor combination. We reason that the clavulanate component of amoxicillin-clavulanate served to inhibit the ESBL, resulting in effective cefdinir activity against most isolates. Clavulanate is very effective in inhibiting ESBLs in vitro (15). In fact, phenotypic detection of ESBLs involves testing of substrate drugs (i.e., cefotaxime and ceftazidime) alone and in the presence of a fixed concentration of clavulanate (5). Markedly increased susceptibility in the presence of the β-lactamase inhibitor provides phenotypic evidence of the production of an ESBL. Cefdinir is an oral extended-spectrum cephalosporin with activity against many members of the *Enterobacteriaceae*, resists hydrolysis by several common β-lactamases, and has excellent urinary penetration (2, 6). Uncomplicated UTIs due to non-ESBL-producing strains treated with cefdinir resulted in a 91.3% clinical cure rate in one study (12). Clavulanate is not available for administration by itself, but coadministration of amoxicillin-clavulanate with cefdinir represents a theoretically attractive option for oral therapy of UTIs due to ESBL-producing organisms. Both cefdinir and amoxicillin-clavulanate achieve high drug levels in urine (amoxicillin-clavulanate and cefdinir package inserts [http://dailymed.nlm.nih.gov/dailymed/about.cfm]).

Another promising option is fosfomycin, which inhibited 91.3% of the urine CTX-M ESBL-producing isolates in this study. Fosfomycin, a derivative of phosphonic acid, targets bacterial cell wall synthesis. It is well tolerated and can be administered as a once-daily dose (10). The drug's excellent urinary penetration and the rarity of resistance to it in clinical isolates also make it an appealing option for treating outpatient UTIs (11, 20).

Ertapenem, a long-acting parenteral carbapenem, was active against 100% of CTX-M- and SHV- or TEM-producing isolates in this study. A previous study revealed 100% susceptibility to ertapenem of ESBL-producing *Enterobacteriaceae* (including *E. coli*, *Proteus mirabilis*, and *Klebsiella* species) isolates causing community-onset urinary infections with only slight increases in the MIC_{50} for strains that produced ESBLs (0.03 μg/ml) compared to that for strains that did not produce an ESBL (0.015 μg/ml) (1). Another study in which outpatient urinary ESBL-producing *E. coli* isolates retained 100% susceptibility to ertapenem (with a MIC_{90} of 0.06 μg/ml) supports our findings as well (24). Ertapenem's stability to hydrolysis by several β-lactamases; its long half-life, which allows for once-daily dosing; and its ability to concentrate in the urine make it another potential option for outpatient therapy (24).

Appropriate outpatient treatment options targeting urinary ESBL-producing *E. coli* strains are increasing in importance. The correct choice of empirical and targeted antibiotic therapy is especially important in preventing progression to more serious infections such as bacteremia, which is associated with increased mortality. Further, it is important that laboratories test for ESBL producers from outpatient urine cultures and test relevant drugs to assist with culture-directed therapy of proven infections due to ESBL producers. Our data indicate that the novel cefdinir-plus-amoxicillin-clavulanate combination, fosfomycin, and the once-daily carbapenem ertapenem are promising treatment options for outpatient UTIs due to CTX-M ESBL-producing *E. coli* strains. Clinical studies are needed to explore the utility of these treatment options.

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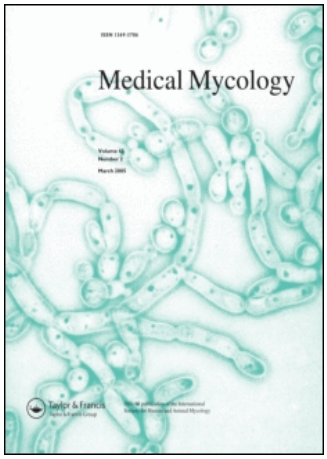

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Lecythophora mutabilis prosthetic valve endocarditis in a diabetic patient

Marci Drees [a]; Brian L. Wickes [b]; Mamta Gupta [c]; Susan Hadley [a]
[a] Division of Geographic Medicine & Infectious Diseases, Department of Medicine, Tufts-New England Medical Center, Boston, Massachusetts, USA
[b] Department of Microbiology and Immunology, The University of Texas Health Sciences Center at San Antonio, San Antonio, Texas, USA
[c] Department of Pathology, Tufts-New England Medical Center, Boston, Massachusetts, USA

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Case Report

Lecythophora mutabilis prosthetic valve endocarditis in a diabetic patient

MARCI DREES*, BRIAN L. WICKES†, MAMTA GUPTA‡§ & SUSAN HADLEY*

**Division of Geographic Medicine & Infectious Diseases, Department of Medicine, Tufts-New England Medical Center, Boston, Massachusetts, †Department of Microbiology and Immunology, The University of Texas Health Sciences Center at San Antonio, San Antonio, Texas, and ‡Department of Pathology, Tufts-New England Medical Center, Boston, Massachusetts, USA*

While dematiaceous (dark-walled) fungi are ubiquitous in the environment, their involvement in invasive human infections has rarely been reported. However, these organisms have been identified as potential emerging pathogens, particularly among immunocompromised hosts. We describe a diabetic patient with *Lecythophora mutabilis* prosthetic valve endocarditis who was treated surgically, as well as with amphotericin B lipid complex and voriconazole, which were subsequently followed by prolonged voriconazole suppressive therapy. To the best of our knowledge, our patient is the first reported survivor of *L. mutabilis* prosthetic valve endocarditis.

Keywords *Lecythophora mutabilis*, endocarditis, dematiaceous fungi, phaeohyphomycosis

Introduction

The dematiaceous fungi, e.g., *Lecythophora mutabilis*, are characterized by the presence of melanin or melanin-like pigments in the cell walls of their hyphae, conidia, or both. Melanin is considered a virulence factor due to its antioxidant and other properties [1,2]. With at least 109 species from 60 genera [3], these saprophytic fungi are widely distributed in the environment and are found in soil, wood, vegetative matter, and polluted water. These fungi have undergone considerable reclassification over time. Obsolete synonyms for *Lecythophora mutabilis*, include *Phialophora mutabilis* and *Margarinomyces mutabilis*. Dematiaceous fungi have long been recognized as causative agents of mycetoma and chromoblastomycosis, but cases of invasive disease (disseminated phaeohyphomycosis) appear to be increasing, as these organisms take advantage of the biologic niche provided by increasing numbers of immunocompromised patients [1].

Case report

A 58-year-old man with coronary artery disease, diabetes, chronic obstructive pulmonary disease (COPD), congestive heart failure (CHF), chronic renal insufficiency and critical aortic stenosis underwent bypass grafting and aortic valve replacement with a porcine tissue valve in July 2004. In October 2004, an automatic cardiac defibrillator was implanted, but the surgery was complicated 10 days later by a methicillin-resistant *Staphylococcus aureus* (MRSA) pocket infection and bacteremia. The device was explanted and he completed a 6-week course of appropriate antibiotics. Between November 2004 and February 2005, the patient was hospitalized multiple times at another facility for fever and shortness of breath, attributed to COPD exacerbations and treated with antibiotics and steroids. Blood, urine, and pleural fluid cultures

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§Current affiliation: Department of Pathology, Dartmouth Hitchcock Medical Center, Lebanon, NH, USA.
Correspondence: Susan Hadley, Tufts-New England Medical Center, 750 Washington Street, #041, Boston, MA 02111, USA. Tel: +1 617 636 1567; Fax: +1 617 636 8525; E-mail: shadley@tufts-nemc.org

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remained negative. To evaluate persistent fever, in February 2005 a transesophageal echocardiogram (TEE) and tagged white blood cell scan were performed, both of which were negative. One month later, he was afebrile but was noted to have a leukocyte count of 16.4×10^3 cells/μl, with 76% neutrophils and 6% eosinophils, and an elevated erythrocyte sedimentation rate and C-reactive protein. Blood cultures obtained at this time remained negative.

Approximately three weeks later, the patient presented again to the same facility with fever, chills, pleuritic chest pain. A TEE failed to reveal the presence of vegetation. He developed electrocardiographic changes and worsening CHF and was transferred to our facility. A repeat TEE revealed a large echodensity encasing and restricting the aortic valve leaflets and obstructing the aortic outflow tract. The patient was taken for emergency valve replacement, and was found to have a 4–5 cm lobulated dense mass originating from the entire aortic valve prosthesis (Fig. 1). Tissue Gram stain revealed 3+ neutrophils and 2+ fungal elements. All blood cultures obtained at both facilities remained negative.

Histologic examination of the prosthetic aortic valve revealed innumerable non-pigmented, septate, hyphal fungal elements (Fig. 2) with bulbous swelling and branching reminiscent of *Aspergillus* or *Pseudallescheria* species. The valve was cultured on blood, chocolate and EMB agars without any growth. However, low, waxy colonies with feet and a few white aerial hyphae were evident within 48 h of inoculation on brain heart infusion (BHI) and Sabouraud dextrose agar (SAB) in tubes and SAB in plates. The colonies matured within five days, becoming salmon-colored

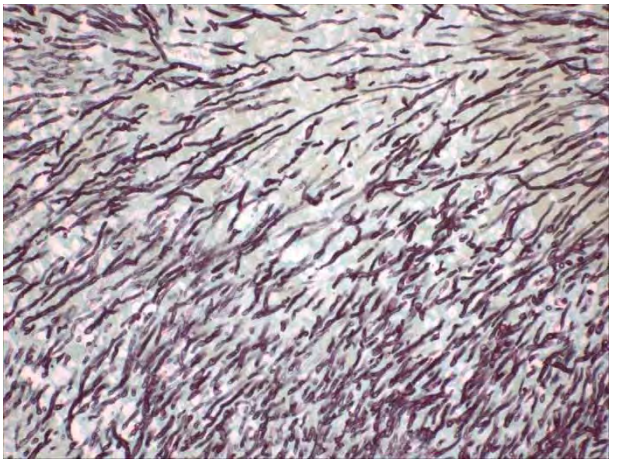

Fig. 2 Gomori methenamine silver (GMS) stain of aortic valve, showing innumerable fungal elements due to *Lecythophora mutabilis*. (See color online.)

and acquiring a central brown pigmentation (Fig. 3). Lactophenol cotton blue (LCB) scotch tape preparation showed development of long thin, hooked conidia, some of which showed swelling with long thin extensions, rounded ends and large vacuoles.

Biochemical testing did not identify the organism, and samples were sent to the Fungus Testing Laboratory at the University of Texas Health Sciences Center (UTHSCSA) for further identification. At UTHSCSA it was provisionally identified as *Lecythophora mutabilis* based on its morphology on potato flakes agar, with cream to yellowish colonies that darkened with a yellow-orange periphery and production of dark chlamydoconidia [4]. Confirmatory DNA sequencing of the D1/D2 region (large subunit RNA gene) was performed at the UTHSCSA Advanced Nucleic Acids

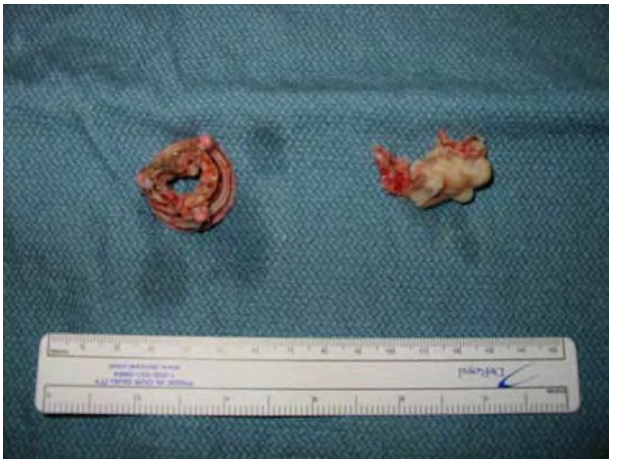

Fig. 1 Prosthetic aortic valve (left) and large, obstructing vegetation (right). (See color online.)

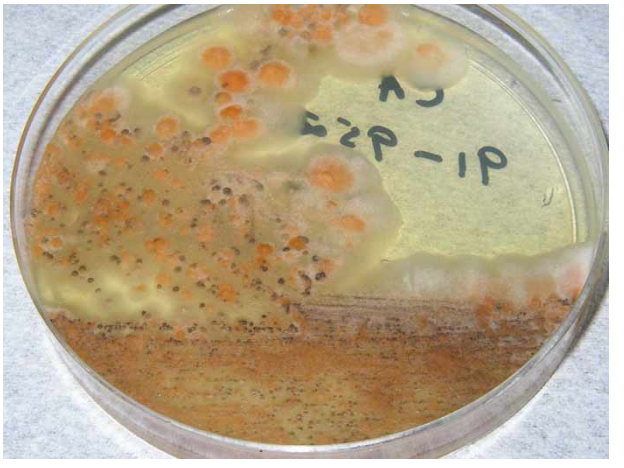

Fig. 3 Appearance of mature colonies *Lecythophora mutabilis* on Sabouraud's dextrose agar. (See color online.)

Core facility. A BLASTn search of the NCBI database found a match of 575/575 bases (100% identity) for *L. mutabilis*. The sequence was deposited in Genbank under accession number EF517490. Fungal susceptibility testing was performed in accord with the Clinical Laboratory Standards Institute M-38-A broth microdilution method [4], and minimum inhibitory concentrations (MIC) at 48 h revealed susceptibility to amphotericin B (MIC 0.25 μg/ml), caspofungin (MIC 2 μg/ml), voriconazole (MIC 0.125 μg/ml), and posaconazole (0.03 μg/ml). The isolate was sent for deposit to the University of Alberta Microfungus Collection and Herbarium (UAMH), Edmonton, Alberta, Canada, where it was accessioned as UAMH 10554.

Postoperatively, while fungal identification and susceptibilities were pending, the patient was treated with amphotericin B lipid complex (ABLC) and oral voriconazole. Dose adjustment was required for worsening renal function while on ABLC. He remained afebrile and was discharged home on postoperative day 32. After a 6-week course, ABLC was discontinued due to renal toxicity and the availability of antifungal sensitivities, and the patient was continued on oral voriconazole. Subsequently, his recovery was complicated by an MRSA catheter-related bloodstream infection, followed by recurrent endocarditis and perivalvular abscess. Blood cultures revealed MRSA only and he was not deemed a candidate for further surgery, limiting our ability to rule out persistent *L. mutabilis* infection. No embolic or metastatic infections occurred during either episode of endocarditis. After prolonged intravenous anti-staphylococcal and antifungal treatment, he remains well, as of 23 months postoperatively, on chronic suppressive therapy with minocycline and voriconazole.

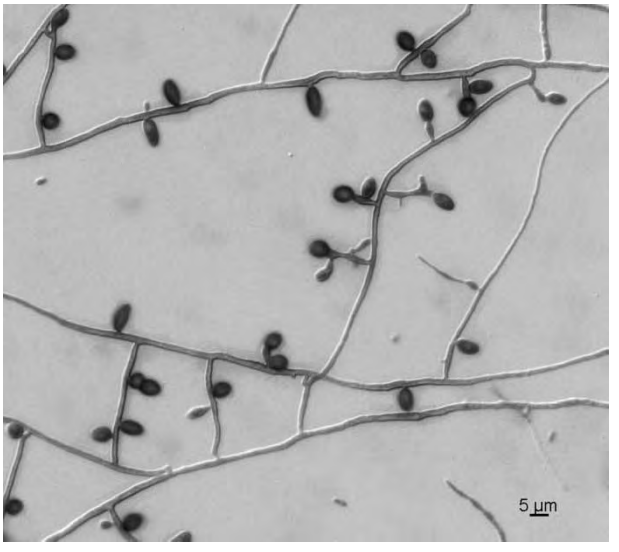

Fig. 4 Pigmented chlamydospores characteristic of *Lecythophora mutabilis*. Published with permission by Lynne Sigler, MSc, Curator, University of Alberta Microfungus Collection and Herbarium.

Discussion

Endocarditis and other invasive infections due to dematiaceous fungi have been reported in a variety of hosts, including premature neonates [5], diabetics [6], bone marrow transplant recipients [7], hemodialysis patients [8], and solid organ transplant recipients [9]. A review [1] of 72 reported cases of disseminated phaeohyphomycosis revealed heart valve infections in 21 (29%) and overall mortality of 79%. In a retrospective review [10], 152 reported cases of fungal endocarditis cases were identified during 1995–2000, of which 39 (26%) were due to molds. Six (15%) of the latter cases were due to dematiaceous fungi. Notably, the mortality rate for mold-related endocarditis was significantly higher than that due to yeast (82% vs. 40%).

L. mutabilis has been described as a cause of invasive disease in humans, including endophthalmitis [11,12] and relapsing fungal peritonitis in a peritoneal dialysis patient [13]. Endocarditis due to *L. mutabilis* has been described only twice previously, both in the 1970s and involving prosthetic valves. The first case was a 56-year-old woman with a porcine mitral valve [14], and the second a 47-year-old man with mechanical mitral and aortic valves [15]. Neither patient was known to be immunocompromised and, similar to our patient, both had negative blood cultures and large, obstructing vegetations. Interestingly, all three patients were noted to have peripheral eosinophilia (which was transient in our patient's case), an uncommon finding in fungal infections. It has been suggested that phaeohyphomycosis be considered in the differential diagnosis of eosinophilia [1]. Seeing as both of these previous cases were fatal, our patient, to the best of our knowledge, is the first reported survivor.

The source of our patient's infection remains unclear. Possibilities include environmental contamination at the time of surgery, contamination of the valve at the site of manufacture, or environmental exposure of the patient after surgery. No additional cases of *L. mutabilis* have been reported from our institution. The patient used well water at home; samples were obtained but *L. mutabilis* was not isolated. No other environmental risk factors were identified.

Treatment options for invasive infections with dematiaceous fungi can be quite limited. While our patient's *L. mutabilis* isolate appeared quite sensitive to a variety of antifungal agents, many related fungi are resistant to amphotericin B, often considered the ideal empiric and definitive treatment for fungal infections [1]. Voriconazole has been used successfully to treat *L. mutabilis* endophthalmitis [12] and infections caused by other dematiaceous fungi [16–19], but treatment failures have also been reported [20–22]. We elected to treat our patient with both ABLC and voriconazole because of the severity of his infection and delay in acquiring antifungal sensitivities. However, to date there has been limited data supporting the use of multiple antifungal agents. In addition, the relationship between *in vitro* antifungal activity and clinical efficacy remains unclear. When possible, surgical as well as medical management is likely required. Because of the recurrent episode of endocarditis, during which *L. mutabilis* could not be definitively ruled out, we elected to maintain our patient on chronic suppressive oral voriconazole. This has now continued for nearly 2 years, with no evidence of recurrence and no adverse effects thus far.

The dematiaceous fungi, including *L. mutabilis*, are capable of causing life-threatening, invasive infections even in patients who are not traditionally considered highly immunocompromised. Our patient's risk factors for fungal disease included significant antibiotic exposure and intermittent steroid use, but diabetes was his only active immunocompromising condition. Clinicians should remain aware of these emerging pathogens, as their diagnosis may be difficult to ascertain using traditional methods. Blood cultures frequently remain negative and, for endocarditis, repeated echocardiography may be required. Intensive and prolonged microbiological effort is required to identify these fungal pathogens. Broad antifungal therapy and early surgical management likely contributed to our patient's survival.

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Potential conflicts of interest

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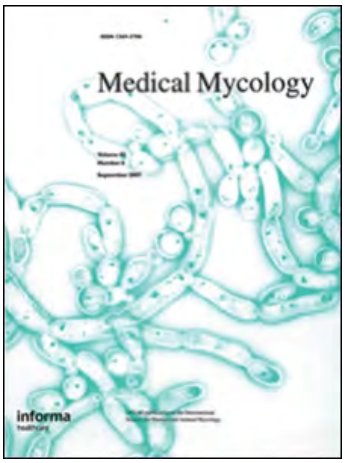

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Pulmonary Phialemonium curvatum phaeohyphomycosis in a Standard Poodle dog

D. A. Sutton [a]; B. L. Wickes [b]; E. H. Thompson [a]; M. G. Rinaldi [a]; R. M. Roland [c]; M. C. Libal [d]; K. Russell [d]; S. Gordon [c]
[a] Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA [b] Department of Microbiology, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA [c] Department of Small Animal Clinical Science, Texas A & M University, College Station, Texas, USA [d] Department of Pathobiology, College of Veterinary Medicine and Biomedical Science, Texas A & M University, College Station, Texas, USA

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Pulmonary *Phialemonium curvatum* phaeohyphomycosis in a Standard Poodle dog

D. A. SUTTON*, B. L. WICKES†, E. H. THOMPSON*, M. G. RINALDI*, R. M. ROLAND‡, M. C. LIBAL§, K. RUSSELL§ & S. GORDON‡

**Fungus Testing Laboratory, Department of Pathology and, †Department of Microbiology, University of Texas Health Science Center at San Antonio, San Antonio, Texas, ‡Department of Small Animal Clinical Science, and §Department of Pathobiology, College of Veterinary Medicine and Biomedical Science, Texas A & M University, College Station, Texas, USA*

Phialemonium curvatum, frequently misidentified as an *Acremonium* species, is reported here as a new agent of pulmonary phaeohyphomycosis in a Standard Poodle dog, and added as a new species in the genus to cause mycoses in canines. *In vitro* susceptibility data, for both human and animal isolates, suggests resistance to amphotericin B and susceptibility to the triazole agents itraconazole, voriconazole, and posaconazole.

Keywords *Phialemonium curvatum*, canine, systemic phaeohyphomycosis

Introduction

Systemic phaeohyphomycosis, a disease associated with saprobic dematiaceous fungi, has been reported infrequently in the dog. In humans *Cladophialophora bantiana* (synonyms, *Cladosporium trichoides, Cladosporium bantianum, Torula bantiana, Xylohypha bantiana, Xylohypha emmonsii*) is known to be neurotropic, and animals with systemic phaeohyphomycosis also commonly present with neurologic disease. In four reports of systemic *C. bantiana* infection in dogs the animals presented with a clinical history and/or signs of central nervous disease including tetraparesis, neck stiffness, back pain, circling, opisthotonus, nystagmus, protrusion of the nictitating membrane and/or seizures [1–4]. *Ochroconis gallopavum* [5] and *Aureobasidium pullulans* [6] have been isolated from lesions in dogs presenting with ataxia, seizures or 'neurologic dysfunction' and in another case dematiaceous fungi were demonstrated in the brain of an animal presenting with convulsions [7]. Rarely, animals with phaeohyphomycosis present with other primary clinical disease, as in the pug dog with a chronic skin infection and who had a dual systemic infection caused by *Bipolaris spicifera* and *Candida* (*Torulopsis*) *glabrata* [8]. Most cases of systemic phaeohyphomycosis in the dog have been diagnosed at necropsy. In the case reported here, the etiologic agent of pulmonary disease was detected antemortem.

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Correspondence: Deanna A. Sutton, Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas, USA. Tel: +1 210 567 4032. Fax: +1 210 567 4076. E-mail: suttond@uthscsa.edu

Case report

A two-year-old male, castrated Standard Poodle was presented to the Texas A&M University Veterinary Medical Teaching Hospital in August, 2005 for definitive surgical repair of an atrial septal defect. The surgery was successful. Several days post-operatively the dog developed vasculitis, pancreatitis, as well as pneumonia, which were treated with palliative therapy in combination with enrofloxacin and ticarcillin/clavulanic acid. In October, 2005 the dog developed a chylous pleural effusion, which was corrected by surgical ligation of the thoracic duct in November 2005. The chylous effusion was thought to represent a complication of his previous open heart surgery. Following this surgery, the dog developed pneumonia again as well as a serosanguinous pleural effusion. The pneumonia was treated with enrofloxacin as well as amoxicillin/clavulanic acid. The pleural effusion became chronic and frequent thoracocenteses were performed to help control the clinical signs associated with the effusion. In December, 2005 a course of somatostatin was given but failed to resolve the effusion.

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Subsequently immunosuppressive prednisone therapy was initiated. After the addition of prednisone, the dog remained asymptomatic for his pleural effusion for eight months, during which the dose was gradually reduced to an anti-inflammatory dose range and the severity of the effusion was monitored with thoracic radiographs and echocardiography. Pleurocentensis was not performed at anytime during this 8 month period, after which the dog developed dyspnea and a severe suppurative pleural effusion. In addition, right rear lameness developed and a right carpal joint tap revealed septic, suppurative effusion. A urinary tract infection was also documented at this time. The dog was treated with thoracocentesis as needed to control clinical signs of dyspnea and a combination of ticarcillin/clavulanic acid, amoxicillin/clavulanic acid, and enrofloxacin. The prednisone was continued although doses were tapered. Over the next few months the dog presented multiple times with dyspnea due to pleural effusion and thoracentesis was performed. The cytologic examinations of pleural fluid samples varied slightly during this time period with the fluid being classified as either a transudate (low cell counts and low total protein concentration) or a modified transudate (mildly increased cell count or total protein concentration), depending on total nucleated cell counts and total protein concentrations. Several bacterial cultures of the pleural fluid yielded no growth. Given the severity of the recurrent pleural effusion in the face of reducing prednisone doses, and the historical response of the pleural effusion to immunosuppressive doses of prednisone, azothioprine was added to the drug regimen in an attempt to further immunosuppress the patient, and potentially allow the dose of prednisone to be reduced. Azothioprine was not well tolerated and was discontinued. Prednisone was continued at immunosuppressive doses and antibiotic coverage with a combination of clavamox and enrofloxacin were continued. Intermittent pleurocentesis for symptomatic pleural effusion continued although the frequency was somewhat reduced. In October, 2006 the animal was presented again with dyspnea and severe pleural effusion. New skin lesions had also developed near the right carpal footpad and over both tarsi. Cytologic examination of pleural fluid revealed a modified transudate/hemorrhagic effusion and cytologic examination of the footpad lesion revealed mild inflammation with intracellular fungal elements present. In addition, multiple lesions suggestive of dermatophytosis developed on the skin of the inguinal area. Three bacterial cultures and one fungal culture were inoculated with pleural fluid samples (collection dates 27 October 2006, 14 November 2006, 22 November 2006 and 3 November 2006 respectively). In addition, fungal cultures were started with samples from the footpad and tarsal lesions (collection date 14 November 2006). Pleural fluid samples for bacteria were inoculated onto trypticase soy agar with 5% sheep blood, MacConkey's agar, and into tryptose broth (Becton Dickinson, Sparks, MD) and incubated at 37°C in 5% CO_2 for up to 5 days. The first two pleural fluid samples yielded no bacterial growth however a fungus was isolated on the blood agar plates inoculated with each of these samples after 3 and 4 days of incubation, respectively. The third pleural fluid sample yielded *Staphylococcus aureus* after one day of incubation and a fungus on day 5 of incubation. The fungal isolates cultured from pleural fluid on three separate occasions appeared identical. One pleural fluid sample inoculated onto only Sabouraud dextrose agar ([SDA], (BD, BBL, Sparks, MD) was negative after 18 days incubation at 25°C. *Microsporum gypseum* was recovered in cultures started with the tarsal skin samples. The footpad culture grew a sterile dematiaceous mould that was subsequently identified as a coelomycete morphologically resembling a *Microsphaeropsis arundinis* (distinct from the fungi isolated from the pleural fluid samples). At this time azathioprine was discontinued and the dog was started on amphotericin B. The dog then developed a methicillin-resistant *Staphylococcus aureus* infection. Due to the poor prognosis and deteriorating condition, the dog was euthanized in December 2006. Necropsy was not performed.

Identification of the etiologic agent

Three fungal isolates from pleural fluid samples and the isolate from the footpad were forwarded to the Fungal Testing Laboratory at University of Texas, San Antonio, TX for identification. The pleural fluid isolates were accessioned into their stock collection as UTHSC 06-4324, 06-4325, and 06-4326. The morphologic features of the isolates were examined on in house prepared potato flakes agar (PFA) incubated at 25°C. Growth rate was moderate and after two weeks incubation colonies were white to cream, floccose, effuse, with centrally raised areas. Discrete, moist, salmon to brownish-yellow sporodochial areas (macroscopically visible cushion-like masses of short conidiophores bearing conidia) formed throughout the cultures after 4 weeks incubation (Fig. 1 – taken at 8 weeks). Microscopically, hyaline hyphae produced numerous coils and complex fasicles (bundles of hyphae). Conidiogenous cells consisted primarily of adelophialides (short phialides lacking a basal septum) produced directly on the hyphae (Fig. 2) and from coils.

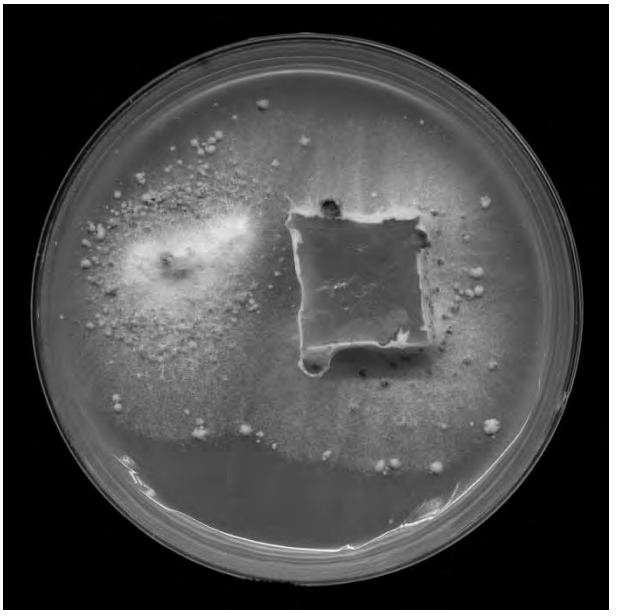

Fig. 1 Potato flakes agar plate, 8 weeks at 25°C, showing area of slide culture preparation on the right, and an undisturbed colony on the left. Salmon to brownish-yellow, moist, raised sporodochial areas are seen throughout the culture.

However longer phialides delimited by a basal septum as seen in *Acremonium* species were also occasionally present. Long, setae-like phialides were also produced from the sporodochia. Slightly allantoid (curved) conidia (1–1.5 × 4.4.5 μm) were borne in mucoid clusters at the apices of these conidiogenous cells. Chlamydoconidia were also present. Based on the features noted above, the isolate was morphologically identified as *Phialemonium curvatum* [9–11].

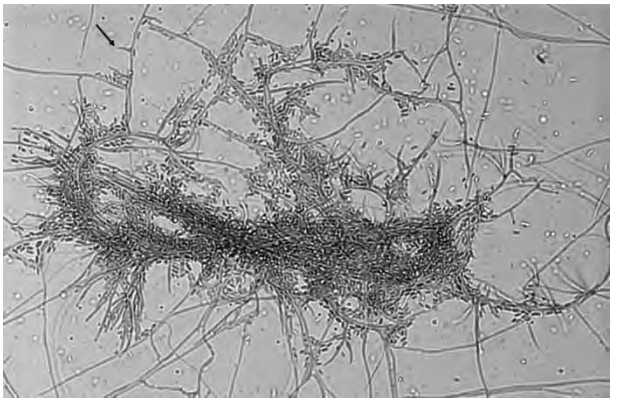

Fig. 2 Microscopic morphology of a young, immature sporodochium after 7 days growth at 25°C on potato flakes agar. Figure depicts short adelophialides (reduced phialides lacking a basal septum), black arrow, as well as longer phialides delimited by basal septa as seen in *Acremonium* species.

One of the isolates, UTHSC 06-4324 (= R-3884) was submitted for molecular characterization to confirm the morphologic identification. DNA was isolated from conidia recovered from a 72 h PDA plate using the Prepman Ultra reagent (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Five microliters each of the supernatant were used in two PCR reactions to amplify the ITS and D1/D2 regions from the rDNA locus. The ITS region was amplified as described using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [12]. The D1/D2 region was amplified using primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3) as described [13,14]. Both PCR reactions were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA) using Triple Master Taq polymerase (Fisher Scientific, Pittsburgh, PA). Amplicons were purified using a Qiaquick PCR purification kit (Qiagen, Inc., Valencia, CA) and then sequenced on both strands at the UTHSCSA Advanced Nucleic Acids Core facility. The data obtained from each sequence were then used to perform BLASTn searches at the NCBI website <http://ncbi.nlm.nih.gov/BLAST/> [15]. Identifications were made at a cutoff of ≥ 98% sequence identity.

The results of the two BLAST searches showed the greatest identity with other sequences deposited from *P. curvatum*. The top three hits for the D1/D2 sequence were *P. curvatum* sequences, each at 99% identity. The top three hits for the ITS search were *P. dimorphosporum*, *P. curvatum*, and *P. curvatum*, each at 99% identity. Since *P. dimorphosporum* is a synonym of *P. curvatum* [16], the sequence identity of the isolate was assigned as *P. curvatum*. The case isolate UTHSC 06-4323 (= R-3884) has been deposited in the University of Alberta Microfungus Collection under the accession number UAMH 10825. The nucleotide sequence data has been deposited into GenBank under the accession numbers EU035984 (ITS) and EU035985 (D1/D2).

The footpad isolate was accessioned as UTHSC 06-4327. After one month incubation at 25°C on a variety of media prepared in-house including PDA, V-8 agar, and carnation leaf agar [17], rare pycnidial structures developed. Conidia were narrow-cylindrical, 1–1.5 × 4 μm, individually subhyaline, but dark in mass. Based on these features the isolate morphologically resembled the coelomycete *Microsphaeropsis arundinis*. The recovery of this organism from the footpad, while potentially significant for localized infection at this site [18,19], was not contributory to systemic fungal

disease. No additional testing was performed on this isolate.

In vitro antifungal susceptibility testing

Retrospective antifungal susceptibility testing of *P. curvatum* was accomplished in a macrobroth dilution format in essential agreement with the previously published Clinical and Laboratory Standards Institute document M38-A [20]. Amphotericin B (AMB, Bristol-Meyers, Squibb, New York, NY) and caspofungin (CAS, Merck, Rahway, NJ) were tested in Antibiotic Medium 3 (Difco, Sparks, MD) while 5-fluorocytosine (5FC, Valient, Irvine, CA), fluconazole, voriconazole (FLC, VRC, Pfizer, Inc., New York, NY), itraconazole (ITC, Janssen Pharmaceutica, Piscataway, NJ) and posaconazole (PSC, Schering Plough, Galloping Hill, NJ) were tested in RPMI-1640 (Hardy Diagnostics, Santa Maria, CA). Tubes were incubated at 35°C with endpoints read at 24 and 48 h. The endpoints for AMB were the lowest concentration that inhibited visual growth, while those for 5FC and the triazoles were 80% inhibition compared to the growth control. Caspofungin endpoints were read as minimum effective concentrations (MECs) [21,22]. Results at 24/48 h were as follows in μg/ml: AMB 2/4; CAS 0.25/0.5; 5-FC >64; FLC 8/16; ITC 0.06/0.25; VRC 0.125/0.25; PSC 0.03/0.125.

Discussion

The genus *Phialemonium,* having morphologic features between the genera *Acremonium* and *Phialophora,* currently contains two species, *P. obovatum* and *P. curvatum* [9,16]. *Phialemonium obovatum* produces a distinct, pale green diffusing pigment, has obovate conidia (like an upside-down egg), and has been previously reported in German shepherd dogs causing osteolytic [23] and disseminated disease [24]. To our knowledge, this is the first report of *P. curvatum* in the veterinary literature. As the use of SDA as a sole primary isolation medium is less than optimal for the recovery of filamentous fungi, this and other etiologic agents may be under-diagnosed. Sporodochial-forming *Phialemonium curvatum* isolates were initially recognized in 2004 in human cases of hemodialysis-associated endovascular infection [10]. They have subsequently been seen in cases of endocarditis and endophthalmitis stemming from intracavernous penile autoinjections of contaminated fluids [11,25], and from intra-articular injection of corticosteroids [26]. Isolates are often misidentified as *Acremonium* species based on the overall macroscopic and microscopic similarities of the two genera.

In humans, the formation of phialides and phialoconidia within tissues in the host, termed 'adventitious' conidia by Liu *et al*. [27], appear to facilitate hematogenous dissemination inciting fungemia [16], endocarditis [28,29], and peritonitis [30]. Disseminated disease usually occurs in the setting of immune compromise. The same scenario presumably occurs in dogs. On occasion dogs operated for chylous effusion can develop non-chylous effusion post operatively that responds to immunosuppression suggesting an underlying inflammatory etiology [31]. However, dogs receiving chronic immunosuppressive agents are, like humans, at risk for infections, particularly from a variety of potential fungal pathogens [32,33]. Secondary infections may also be present and, in this case, likely contributed to the severity of the pleural effusion in the later stages of the disease.

Retrospective antifungal susceptibility results for the case isolate were similar to those seen for human isolates. Although there are no defined breakpoints for this organism, elevated MICs for AMB and 5FC suggested resistance. Clinical deterioration while on AMB therapy may support lack of efficacy for this agent. Itraconazole, as well as the newer triazoles PCZ and VCZ, demonstrated low MICs, while the FLZ MIC was somewhat elevated at 16μg/ml. Minimum effective concentrations for CAS were also low at 0.5 μg/ml; a departure from MEC values seen in human isolates.

In conclusion, *Phialemonium curvatum* is reported as a new agent of pulmonary phaeohyphomycosis in a Standard Poodle dog, and is added as a new species in the genus to cause disease in canines. Based on limited data, the triazole drugs ITZ, VRZ, and PCZ would appear appropriate for empiric therapy pending susceptibility test results.

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GUEST COMMENTARY

Sequence-Based Identification of *Aspergillus*, *Fusarium*, and *Mucorales* Species in the Clinical Mycology Laboratory: Where Are We and Where Should We Go from Here?▽

S. A. Balajee,[1]* A. M. Borman,[2] M. E. Brandt,[1] J. Cano,[3] M. Cuenca-Estrella,[4] E. Dannaoui,[5] J. Guarro,[3] G. Haase,[6] C. C. Kibbler,[7] W. Meyer,[8] K. O'Donnell,[9] C. A. Petti,[10] J. L. Rodriguez-Tudela,[4] D. Sutton,[11] A. Velegraki,[12] and B. L. Wickes[13]

Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, Georgia[1]; Mycology Reference Laboratory, Health Protection Agency, Bristol, United Kingdom[2]; Unitat de Microbiologia, Facultat de Medicina, Universitat Rovira i Virgili, Reus, Spain[3]; Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III, Madrid, Spain[4]; Centre National de Référence Mycologie et Antifongiques, Unité de Mycologie Moléculaire, CNRS URA3012, Institut Pasteur, Paris, France[5]; Institute of Microbiology, University Hospital, RWTH Aachen, 52074 Aachen, Germany[6]; Centre for Medical Microbiology, University College London, London, United Kingdom[7]; Molecular Mycology Research Laboratory, Centre for Infectious Diseases and Microbiology, University of Sydney, Western Clinical School at Westmead Hospital, Westmead Millennium Institute, Westmead, NSW 2145, Australia[8]; Microbial Genomics and Bioprocessing Research Unit, NCAUR-ARS-USDA, Peoria, Illinois[9]; Departments of Medicine and Pathology, University of Utah School of Medicine, Salt Lake City, Utah[10]; Department of Pathology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas[11]; Mycology Laboratory, Department of Microbiology, Medical School, National and Kapodistrian University of Athens, Athens, Greece[12]; and Department of Microbiology and Immunology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas[13]

The identification of fungal species and determination of their significance in the clinical laboratory are complex practices that help establish or exclude a fungal cause of disease. In the past, the clinical mycologist utilized a limited array of phenotypic measurements for categorizing isolates to the species level. This scenario is shifting in favor of molecular identification strategies largely due to a combination of several factors: (i) the changing landscape of epidemiology of medically important fungi, in which novel organisms never before implicated in human infection are being reported from clinical samples (10, 41); (ii) reports of species-specific differences in antifungal susceptibilities of these newly recognized fungi (4, 10, 41); (iii) numerous studies demonstrating that morphology alone may not be a sufficiently objective method for species determination (7, 8, 10, 23, 41); and (iv) a growing scarcity of bench scientists and microbiologists trained in traditional mycology. With the increasing incidence of fungal infections and reports of invasive fungal infections in nontraditional populations, such as patients with critical illnesses, the onus is on the clinical microbiologist/mycologist to return a timely and accurate identification. Molecular methods are rapid with a turnaround time of about 24 h from the time of DNA extraction, yield results that are objective with data portable between labs, and could be more economical in the long run.

Few topics are more controversial or evoke such a passionate response as the term "species" to a mycologist. Molecular studies have demonstrated that a strategy where multiple genes (or portions thereof) are sequenced and the resultant data are analyzed by phylogenetic methods is a robust strategy for fungal species recognition. This concept, known as phylogenetic species recognition (PSR) (40), has been used successfully to define species in the genera *Fusarium* and *Aspergillus* (8, 23, 29, 31, 32). The advent of PSR has greatly clarified the taxonomy of these genera and as such is a powerful tool for fungal species delimitation. However, this methodology is expensive and requires phylogenetic expertise, which may be limiting factors in clinical microbiology laboratories. In reality, once a species has been delimited by PSR using several robust loci, sequence diversity within the species is known, and on the basis of this knowledge, comparative sequence analyses from a single locus can be used for rapid species identification. "Cutoff scores," which are dependent on genetic diversity within and between sibling species, can then be provided.

Thus, it is important to clarify that our intent in this editorial is to address the practice of species "identification" as applied to a clinical setting and not species "classification" necessary for taxonomic categorization. Although the two terms can be overlapping, the purpose of an "identification" method in a clinical microbiology laboratory is the ability to provide a specific name or epithet to an organism rapidly and with precision, without the complex experimental research or detailed phylogenetic analyses vital for a taxonomic "classification" scheme. Such specific information can then be used by the physician in a decision-making algorithm that can guide patient management.

The field of medical mycology has embraced molecular methods of identification, resulting in the exploration of numerous potential targets, an explosion in the number of sequences from these loci, and recognition of previously un-

* Corresponding author. Mailing address: Mycotic Diseases Branch, Centers for Disease Control and Prevention, Mail Stop G 11, 1600 Clifton Road, Atlanta, GA 30333. Phone: (404) 639 3337. Fax: (404) 639-3546. E-mail: fir3@cdc.gov.

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known fungal species adding to the already staggering fungal diversity. On the other hand, this practice may have opened up a number of possibilities, at least from the perspective of a mycologist in a routine microbiology laboratory, resulting in considerable uncertainty about the best possible molecular method to obtain a species identification. Realizing this, a consortium of international experts was assembled as an International Society for Human and Animal Mycology (ISHAM; www.isham.org) working group on fungal molecular identification. With the goal of supporting clinical laboratories in their efforts to identify fungal species from culture by using molecular methods, the ISHAM working group agreed to begin by focusing on molecular strategies available for medically important fungi of the genera *Aspergillus* and *Fusarium* and the order *Mucorales* (Zygomycota). The advantages and limitations of these methods are discussed, and the recommendations of this working group are presented in this editorial.

COMPARATIVE SEQUENCE IDENTIFICATION STRATEGY

Today, comparative sequence-based identification strategies can be considered the new "gold standard" for fungal species identification (39). This method is based on PCR amplification of a selected region of genomic DNA (target locus), followed by sequencing of the resulting amplicon(s). Once a consensus sequence is obtained, it can be queried against a database library and evaluation for species identification can be performed by generating dendrograms, examining percent similarity/percent dissimilarity, or executing more sophisticated phylogenetic analyses. The current approach in clinical laboratory practice is to interpret sequence comparison results by generating a percent identity score, which is a single numeric score determined for each pair of aligned sequences and which measures the number of identical nucleotide matches in relation to the length of the alignment. Cutoff scores for species identification are arbitrary, and the scores can vary depending on numerous factors including the quality of the sequence, the number and accuracy of existing database records from the same species and locus, the length of the sequence fragment, and the software program employed. At present, there is no definitive study describing an absolute cutoff for same-species identity across the fungal kingdom and no consensus definition exists on how to define a species using such comparative sequence methodologies.

The success of a comparative sequencing strategy for the identification of a wide range of clinical fungi lies mainly in the choice of the appropriate locus. The gene target should be orthologous (i.e., evolved by common descent), having a high level of interspecies variation combined with low levels of intraspecific variation, and ideally should not undergo recombination. In addition, the target must be easy to amplify and sequence using standardized "universal" primer sets. Finally, the amplified DNA fragment should be within the size range obtainable with the most commonly used automated DNA sequencers (~600 to 800 bp) and easily aligned with a sequence database for comparison. Does such a utopian locus exist?

Multiple studies have demonstrated that comparative sequence-based identification using the nuclear ribosomal internal transcribed spacer (ITS) region (ITS1, 5.8S rRNA, and ITS2) located between the nuclear small- and large-subunit rRNA genes (43) could be employed for species complex-level identification of *Aspergillus* (21) and most *Mucorales* (37) species and for identification within some species complexes of *Fusarium* (Fig. 1) (31, 44). The ITS region satisfies most of the aforementioned requirements of a "universal" marker since this region can be reliably amplified for most fungi, is conserved, is present as multiple copies in the fungal genome, yields sufficient taxonomic resolution for most fungi, and has the additional advantage that the GenBank(http://www.ncbi.nlm.nih.gov), European Molecular Biology Laboratory nucleotide sequence database (http://www.ebi.ac.uk/embl/), and DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/) contain a large number of sequences from this locus, enabling a ready comparison of the sequence from an unknown isolate.

There is considerable consensus regarding the use of ITS sequencing as the initial step in mold identification. An international *Aspergillus* working group recently recommended the use of the ITS region for subgenus/section-level identification for the genus *Aspergillus* (9). Also, the International Subcommission on Fungal Barcoding has proposed the ITS region as the prime fungal barcode or the default region for species identification (http://www.allfungi.com/its-barcode.php).

Significant disadvantages of the ITS region include (i) insufficient hypervariability to distinguish the various species in the *Aspergillus* sections and *Fusarium* species complexes; (ii) its failure to distinguish between closely related species (sibling species) because of insufficient nucleotide differences, for example, *Aspergillus lentulus* and *Aspergillus fumigatus*; and (iii) problems with the reliability of the ITS sequences deposited in the reference databases (e.g., GenBank/EMBL/DDBJ) (26).

Comparative sequence-based identification strategies can be meaningful only with the availability of well-curated, robust, and reliable databases that are populated with sequence data from type or reference strains (where possible), have been rigorously validated in terms of their nomenclature, and include sequences from a wide variety of target species. The most widely used database is GenBank, which contains a huge number of sequences, but these are combined with unedited and nonvalidated information, which may be updated and corrected only by the original submitter. Errors in fungal sequences within GenBank have been found to be as high as 20% (26). Despite calls for the process to be changed, to allow for third-party revision (11), there seems little prospect of this in the near future (34). On the other hand, smaller databases, such as those provided with commercial sequence-based identification systems, are often inadequate because of their lack of breadth (omitting many, often important species) and depth (containing few representatives of the same species) (19). To overcome these problems, specific sequence databases for particular groups of fungi, based on quality-controlled sequences, have been created mainly for plant-pathogenic, industrially important, and ectomycorrhizal ascomycete and basidiomycete fungi, e.g., *Fusarium* spp. (FUSARIUM-ID v. 1.0 [17]; http://fusarium.cbio.psu.edu), *Phaeoacremonium* spp. (http://www.cbs.knaw.nl/phaeoacremonium/biolomics.aspx), and *Trichoderma* spp. (http://www.isth.info/morphology.php), and mycorrhizal fungi (UNITE; http://unite.ut.ee/). Two ITS databases for medical fungi are available through the CBS Fungal Biodiversity Centre and at the Westmead Millennium Institute, University of Sydney (curated database; http://www.mycologylab.org

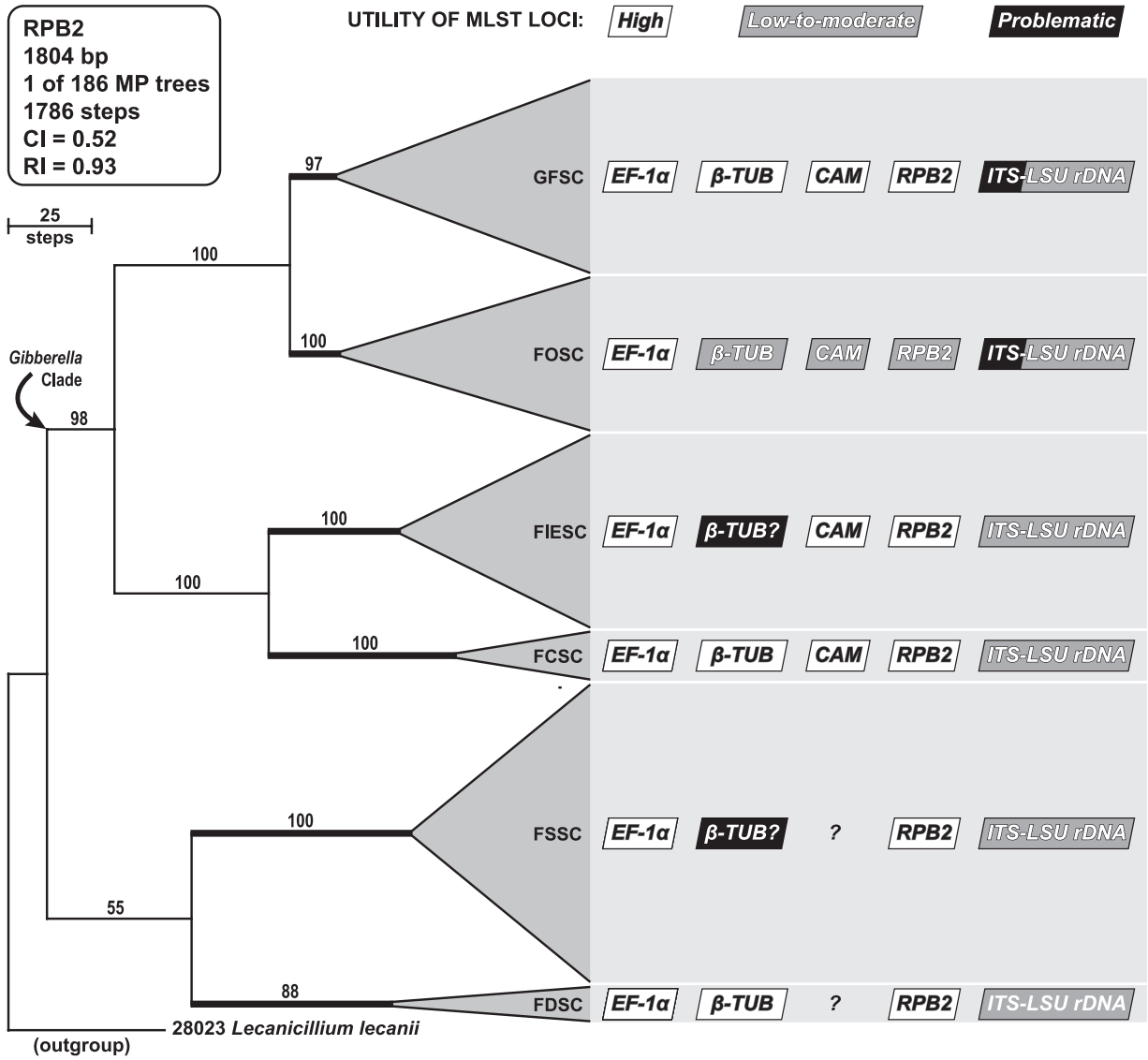

FIG. 1. RNA polymerase II second largest subunit (*RPB2*) phylogeny of *Fusarium* (modified from Fig. 1 in reference 30), showing the utility of DNA sequence data from various loci for resolving at or near the species level within six medically important species complexes. GFSC, *Gibberella fujikuroi* species complex; FOSC, *Fusarium oxysporum* species complex; FIESC, *Fusarium incarnatum-equiseti* species complex; FCSC, *Fusarium chlamydosporum* species complex; FSSC, *Fusarium solani* species complex; FDSC, *Fusarium dimerum* species complex. Loci include *EF-1*α (translation elongation factor), β-*TUB* (β-tubulin), and *CAM* (calmodulin). Numbers above the internodes represent the frequency (%) with which they were recovered from 1,000 bootstrap replicates of the data. A sequence of *Lecanicillium lecanii* was used to root the phylogeny.

/biolomicsid.aspx) (W. Meyer et al., unpublished data). Ideally, the mycology community needs to find a way of combining high-quality sequence and available species data present in numerous reference and research laboratories around the world.

SPECIES IDENTIFICATION IN *ASPERGILLUS*, *FUSARIUM*, AND THE *MUCORALES*

***Aspergillus* species.** Taxonomically, the genus *Aspergillus* is divided into seven subgenera, which are further divided into several "sections"—for example, subgenus *Fumigati* encompasses two sections: *Fumigati* and *Cervini* (24). Clinically relevant aspergilli are represented within several sections of the seven subgenera. For instance, the medically important species *A. fumigatus* and other relatives less commonly implicated in human infections such as *Neosartorya fischeri* and *A. lentulus* fall within section *Fumigati* of the genus *Aspergillus*. Given that this classification scheme is unique to the genus *Aspergillus*, it is important to recognize that there can be two levels of identification: (i) identification to a given species complex, e.g., discrimination of *A. fumigatus* complex (subgenus *Fumigati*, section *Fumigati*) from *Aspergillus flavus* complex (subgenus *Circumdati*, section *Flavi*), and (ii) identification of species within a section, e.g., discrimination of *A. fumigatus* from *A. lentulus* (both members of the section *Fumigati*, subgenus *Fumigati*). Employing comparative sequence analysis of the ITS region, one can rapidly and unquestionably place an *Aspergillus*

isolate within the respective sections, for instance, *Aspergillus ustus* (section *Usti*) or *Aspergillus terreus* (section *Terrei*) (21). In contrast, species identification within a given *Aspergillus* section, for instance, identification of the various species within section *Usti* (i.e., *Aspergillus calidoustus*, *A. ustus*, and *Aspergillus pseudodeflectus*), can be challenging given that the ITS region has few sites that are variable enough for this degree of resolution. In addition, several aspergilli have overlapping morphological features rendering phenotypic identification methods inadequate. Numerous studies have demonstrated that comparative sequence analyses of protein coding regions such as those for β-tubulin, calmodulin, and rodlet A can identify species within sections *Fumigati*, *Usti*, *Nigri*, and *Terrei* (4, 7, 8, 23, 36, 41).

Recognizing the growing role of molecular methods in *Aspergillus* species identification, an international *Aspergillus* working group (9) proposed the following recommendations: (i) the term "species complex" as an alternative to "section," (ii) use of sequences from the ITS region for identification of *Aspergillus* isolates to the species complex level, and (iii) comparative sequence analyses of the β-tubulin region for species identification within a complex. This recommendation can be advantageous to clinical laboratories that rely on comparative sequence analyses of the ITS region (which are not variable enough for species identification within a section) and/or morphology for species identification (where overlapping morphologies can hinder resolution of species within the sections) as they can report the identification of an unknown organism to species complex, for instance, *A. terreus* complex. Thus, the term "complex" in such an identification scheme would indicate the placement of the isolate within a species complex but does not identify the isolate to a species within the complex.

***Fusarium* species.** *Fusarium* species have emerged over the past 3 decades as an important genus of filamentous molds causing opportunistic infections in humans (27). Detailed molecular studies employing sequences of multiple loci such as elongation factor 1α (*EF-1α*) (17), β-tubulin (β-*TUB*), calmodulin (*CAM*), and RNA polymerase II second largest subunit (*RPB2*) (Fig. 1) and subsequent phylogenetic analyses of medically important fusaria have revealed the presence of multiple cryptic species within each morphologically recognized "morphospecies." For instance, *Fusarium solani* actually represents a complex (i.e., *F. solani* species complex) (Fig. 1) of over 45 phylogenetically distinct species of which at least 20 are associated with human infections (31, 44). Similarly, members of the *Fusarium oxysporum* species complex are phylogenetically diverse (31, 44), as are members of the *Fusarium incarnatum-equiseti* species complex and *Fusarium chlamydosporum* species complex (Fig. 1) (30; K. O'Donnell, unpublished data). Cases involving the latter two complexes are typically reported as the polytypic morphospecies *F. incarnatum*/*Fusarium semitectum*/*F. equiseti* and *F. chlamydosporum*, respectively (38). Available data clearly demonstrate that sequences from the nuclear ribosomal ITS region and domains D1 and D2 of the 28S ribosomal DNA (rDNA) large subunit are too conserved to resolve most clinically important fusaria at the species level (31, 44), despite reports to the contrary (16, 20). Moreover, use of the ITS rDNA within the *Gibberella fujikuroi* species complex and *F. oxysporum* species complex (29) and β-tubulin within the *F. incarnatum-equiseti* species complex and *F. solani* species complex should be avoided due to paralogous or duplicated divergent alleles (32; O'Donnell, unpublished).

Mucorales. Evolutionary relationships of species within the order *Mucorales* (of the division Zygomycota) have been investigated by phylogenetic analyses of nuclear ribosomal 18S and 28S rDNA and translation elongation factor (*EF-1α*) gene sequences and have revealed that species within medically important genera such as *Absidia* and *Mucor* appear to be polyphyletic (i.e., from multiple evolutionary origins) (33, 42). Indeed, a recent taxonomic revision of *Absidia* based on physiological, phylogenetic, and morphological characters has been proposed (22), with reclassification of the human pathogen *Absidia corymbifera* as *Mycocladus corymbiferus* in a new family. Similarly, molecular and physiological data were used to distinguish two species within the morphospecies *Rhizopus oryzae* (1, 2, 35), with the proposal that the fumaric-malic acid-producing species be named *Rhizopus delemar* (2).

Several recent studies have demonstrated the utility of comparative sequence analyses of the nuclear 28S rDNA D1/D2 domains, the ITS region, actin, and partial translation elongation factor (*EF-1α*) gene sequences for resolving at or near the species level within the *Mucorales* (1, 2, 33, 37, 42). Analyses of intra- and interspecies variability of ITS sequences from 54 isolates of *Mucorales* belonging to 16 different species were evaluated recently, and the results support ITS sequencing as a reliable method for the accurate identification of most medically important *Mucorales* to the species level (37). However, it is important to note that some closely related species could not be resolved using ITS sequence data. Similarly, while ITS sequence data can be used for the identification of several *Rhizopus* species (1, 25), they lack sufficient variability to resolve *Rhizopus azygosporus* from *Rhizopus microsporus.* In addition the D1/D2 domains of the 28S rDNA and the high-affinity iron permease 1 gene (*FTR1*) appear to be useful targets for species identification, although the *FTR1* locus could not resolve all of the clinically relevant species within the genera *Rhizomucor* and *Mucor* (28). Thus, it is readily apparent that sequencing of more phylogenetically informative gene targets will be required for certain *Mucorales* and that phylogenetic analyses of several loci will be needed to fully assess species limits within the *Mucorales*.

RELEVANCE OF SPECIES IDENTIFICATION IN THE CLINICAL MICROBIOLOGY LABORATORY

An important issue to be considered when deciding the choice of loci and/or number of loci is the relevance of identifying every unknown isolate to the smallest taxonomic unit. In other words, should a clinical microbiology laboratory strive to identify every isolate to the species level, or is it sufficient to identify isolates to the genus or species complex level? Species-level identification of a fungal isolate recovered from a clinical specimen (especially from a sterile site from an immunocompromised patient) could be important given that species identification of appropriate isolates in high-risk populations may reveal the etiological agent of disease, aid selection and monitoring of antifungal therapy, and support epidemiological investigations leading to effective infection control measures. On the other hand, many sporadic isolates do not represent clinically important disease and it may be wasteful to devote re-

sources to identifying such isolates without an understanding of their role in disease.

After identifying the unknown fungal isolate to the level of a species complex, should the laboratory go further to achieve species identification within a section or complex? This is difficult to answer; nevertheless, each one of us in the clinical microbiology laboratory faces this question every time we recover a fungus from a high-risk patient and/or read a manuscript describing yet another species within the complexes. The clinical significance of identifying isolates to species level, for example, *A. terreus* versus *A. fumigatus* in the genus *Aspergillus*, is evident given the different susceptibilities to the antifungal drug amphotericin B; however, the significance of identifying individual species within the complexes of aspergilli and fusaria and to the species level within the *Mucorales* is not fully apparent at this time. Studies have shown species-specific differences in antifungal susceptibilities within *Aspergillus* section *Fumigati* (4), while other studies have shown little or no difference in antifungal susceptibilities of species within the sections *Usti* (41) and *Terrei* (S. A. Balajee et al., unpublished data). Likewise, there appear to be limited species-specific differences in antifungal susceptibilities within the genus *Fusarium* (3, 6, 31). On the other hand, considerable interspecific variation in antifungal susceptibility of the *Mucorales* to polyenes and azoles has been observed in vitro (14, 15) and in in vivo animal models of zygomycosis (18), suggesting that species identification might be clinically relevant in the future as more active antifungal agents against these organisms become available and as in vitro breakpoints are defined.

Taken together, data regarding differences in pathogenicity and in vivo drug susceptibilities of the various species within *Aspergillus* and *Fusarium* complex do not categorically suggest that identification within these taxa will impact clinical and therapeutic decision making, at least at the present time. However, identification to species/strain level could inform the epidemiology of fungal infections and can be critical in outbreak investigations (12, 30). Accordingly, the decision to identify an unknown isolate to species level within a given section/complex of these genera will be based on the need of the clients whom the microbiology laboratory is serving (high-risk versus low-risk populations), site of isolation of the fungus (sterile versus nonsterile sites), funds, and personnel available.

CLSI RECOMMENDATIONS FOR FUNGAL SPECIES IDENTIFICATION

At present, DNA target sequencing can provide a quantitative metric to classify fungi; however, sequencing results can create laboratory uncertainty when assigning microorganisms to their appropriate taxonomical groups. Realizing this, in May 2008, the Clinical and Laboratory Standards Institute (formerly NCCLS) published a document, *Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing*, to address the challenges of sequence analyses in general clinical laboratory practice (13). Specifically, the CLSI guideline provides a systematic and uniform approach to identify fungi by broad-range DNA target sequencing in the clinical laboratory. The document establishes guidelines for primer design, quality control parameters for amplification and sequencing, measurement of sequence quality, and assessment of reference databases. Since consensus has not yet been achieved in multilocus DNA sequencing and since most clinical laboratories do not have the resources to perform such analyses, the guideline focuses on the most commonly used target, the ITS region. For specific taxonomic groups, tables are provided to describe the relative strengths and limitations of individual DNA targets and list alternative DNA targets for those laboratories pursuing further phylogenetic resolution. Finally, the document discusses reporting strategies that are clinically relevant for specific groups of microorganisms. Since our current understanding of the diversity of clinically important phylogenetic species within *Aspergillus* and *Fusarium* is in flux and the biological importance of drawing finer phylogenetic distinctions remains to be determined, the guideline recommends that for certain taxa, clinical laboratories report sequence results for isolates only to the level of genus or species complex.

The CLSI document is largely centered around the ITS region as a target because of the general applicability, research backing, and literature validation of this target. Similarly to CLSI, the ISHAM working group details differences between species complexes and individual species and presents alternative targets that might offer the user more specific species identification in *Aspergillus* and *Fusarium* if such information is needed.

RECOMMENDATIONS OF THE WORKING GROUP

If the goal is to identify an unknown organism with no a priori knowledge, then the ITS region is a reasonable and extensively used choice for species complex identification within the genera *Aspergillus* and *Fusarium* and most species within the *Mucorales*.

Such a consensus on the employment of the ITS region as the default locus for use in the clinical laboratory setting would achieve international consistency in the way that other collaborative initiatives, such as the EORTC/MSG diagnostic criteria for invasive fungal infection (5), have been successful. This consensus should have the effect of enhancing the publication of ITS sequences and focusing commercial efforts on this strategy. Clinical laboratories that have been reluctant to adopt molecular technology in an atmosphere of conflicting opinions and evidence are more likely to implement methodology that has international backing. In addition, the quality of clinical and other research publications would be improved and harmonized based on the use of a universal locus.

This working group acknowledges the known shortcomings of the ITS locus and therefore recommends a staged sequence-based identification strategy (Fig. 2) for identification of aspergilli, fusaria, and the *Mucorales* in a clinical microbiology laboratory. Based on this proposed algorithm, when an unknown fungal isolate is received in a clinical microbiology laboratory, after initial morphological examination the laboratory can pursue morphological or molecular identification methods or choose a combination of the two methods (Fig. 2). When further resolution is required, comparative sequence analyses of one or several protein coding regions can be performed for species-level identification within *Aspergillus* and *Fusarium* complexes.

There is no universal agreement on the identity cutoff values that should be applied for same-species identity, and thus, a

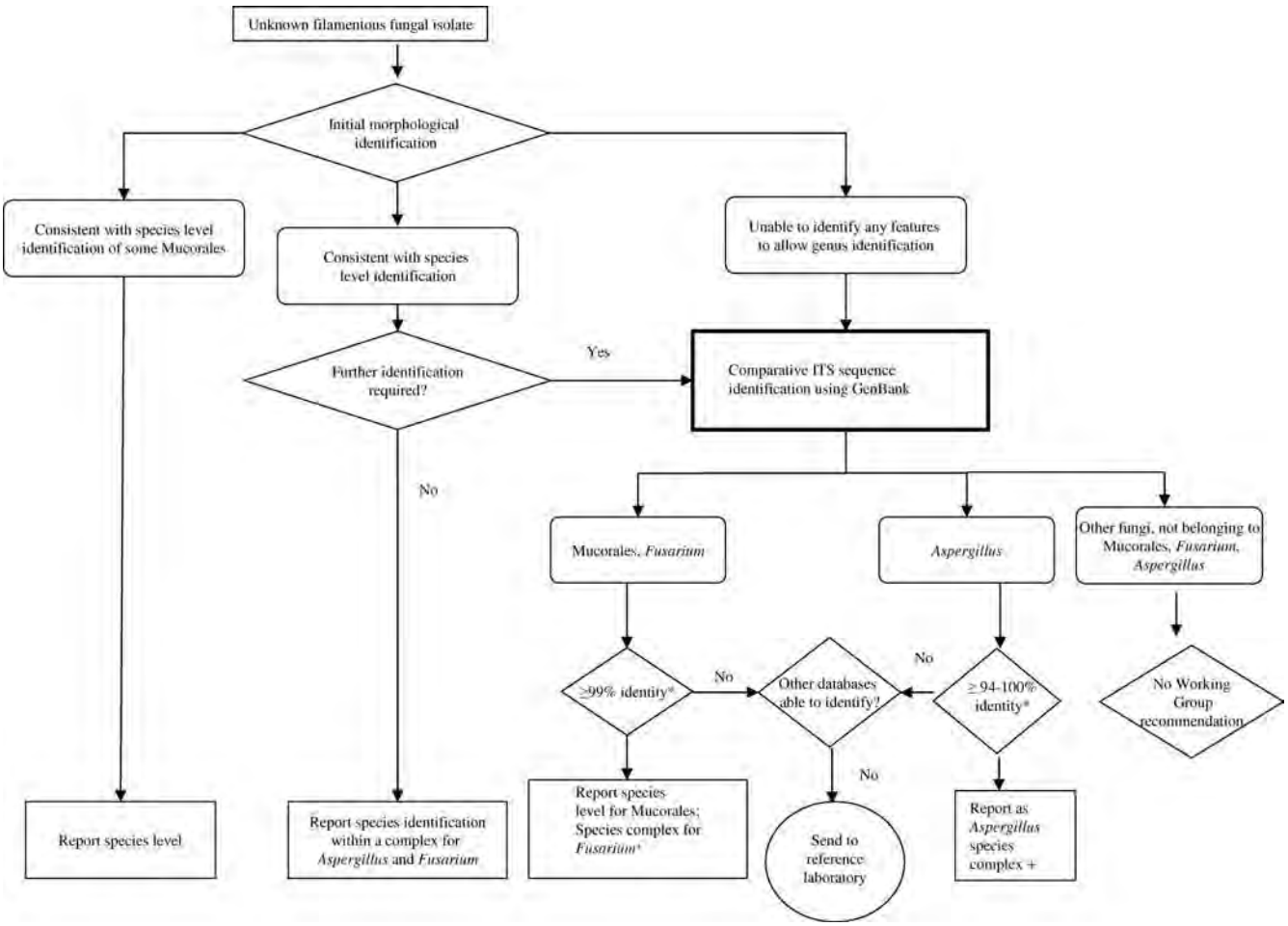

FIG. 2. An algorithm for identification of an unknown filamentous fungal species in a clinical microbiology laboratory. *, many factors affect percent identity scores including quality and length of query sequence and the number and accuracy of existing GenBank records for same species and locus. +, identification to species level within the *Aspergillus* and *Fusarium* complex can be achieved by comparative sequence analyses of protein coding regions.

certain degree of interpretation will be required, at least until the issues already discussed have been resolved. At this time, the CLSI guidelines do not provide cutoff values because, at the time of writing, the available data did not support such cutoff values for fungi. Nevertheless, it will be important for the mycological community to refine guidelines in this difficult area for users in the clinical setting, to ensure consistency of interpretation. Thus far, analyzing ITS sequence data from >600 *Aspergillus* isolates from three different laboratories (S. A. Balajee, W. Meyer, and A. Velegraki, unpublished data) and employing both "in-house" sequence databases and the GenBank/EMBL/DDBJ database for sequence comparison, an identity of 94 to 100% to the respective type/validated strain is proposed for species complex-level identification within the genus *Aspergillus*. For the genus *Fusarium* (Fig. 1) and within most species within the *Mucorales*, we propose that if the ITS sequence of an unknown fungal isolate yields an identity of ≥99% to a type/reference strain, the isolate can be placed within one of six clinically relevant species complexes. When ITS comparative sequence analyses yield ambiguous data, the clinical laboratory may consider sending the isolate to a reference laboratory for identification.

When performing comparative sequence analyses (as outlined in Fig. 2), it is imperative to understand that the percent identity scores generated using GenBank/EMBL/DDBJ are influenced by numerous factors including the quality of the sequence, the number and accuracy of existing GenBank/EMBL/DDBJ records for the same species and locus, and the completeness of the sequence (double-stranded sequence). Importantly, because outputs can be ranked by maximum score, total score, or percent identity and searches can be customized for parameter preferences (i.e., gap penalties and BLAST algorithm), users should take advantage of some of the tutorials and background information prior to performing searches.

In order to improve the accuracy of sequence data, the working group further emphasizes the importance of completing database record fields (especially those of GenBank/EMBL/DDBJ) correctly when submitting sequences for inclusion in these databases. The teleomorph name should be

included if known and available for the organism, and species names should follow guidelines established by the International Code of Botanical Nomenclature. Species identity and sequence accuracy can be confirmed with reference to other sources such as the Centraalbureau voor Schimmelcultures (http://www.cbs.knaw.nl), the UK National Collection of Pathogenic Fungi (http://www.hpacultures.org.uk), and Mycobank (http://www.mycobank.org). It must be remembered, however, that isolates in these collections were mostly identified by morphology alone.

Comparative sequence-based identification is an evolving area of research with the constant addition of new sequences at novel and traditional loci to many different databases. Future studies will be needed to assess the validity of the proposal made in this editorial and to examine its utility in the clinical setting. As noted, the CLSI document is intended to be updated periodically, so that additional research-based evidence can be translated into better-defined algorithms and guidelines of practical benefit. It is an important beginning, and together with the efforts of the ISHAM working group, it should help guide and inform development of this clinical mycology laboratory methodology.

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Isolation and Characterization of a New Fungal Species, *Chrysosporium ophiodiicola*, from a Mycotic Granuloma of a Black Rat Snake (*Elaphe obsoleta obsoleta*)▽

S. Rajeev,[1]* D. A. Sutton,[2] B. L. Wickes,[3] D. L. Miller,[1] D. Giri,[5] M. Van Meter,[6] E. H. Thompson,[2] M. G. Rinaldi,[2] A. M. Romanelli,[3] J. F. Cano,[4] and J. Guarro[4]

Veterinary Diagnostic and Investigational Laboratory, College of Veterinary Medicine, University of Georgia, Tifton, Georgia[1]; Fungus Testing Laboratory, Department of Pathology,[2] and Department of Microbiology and Immunology,[3] University of Texas Health Science Center at San Antonio, San Antonio, Texas; Mycology Unit, Medical School, Rovira i Virgili University, Reus, Spain[4]; Histo-Scientific Research Laboratory, Mount Jackson, Virginia[5]; and Animal Medical Center of Rome, Rome, Georgia[6]

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Isolation and characterization of the new species *Chrysosporium ophiodiicola* from a mycotic granuloma of a black rat snake (*Elaphe obsoleta obsoleta*) are reported. Analysis of the sequences of different fragments of the ribosomal genes demonstrated that this species belongs to the Onygenales and that this species is genetically different from other morphologically similar species of *Chrysosporium*. This new species is unique in having both narrow and cylindrical-to-slightly clavate conidia and a strong, pungent odor.

CASE REPORT

A black, male rat snake (*Elaphe obsoleta obsoleta*) of undetermined age was presented with a history of prolonged anorexia and slow-growing facial masses. The snake was found as an adult at an old home site in an old barn near Sparta, GA, by the current owner, a wildlife educator. The snake had been in his possession for 4 years and was frequently used in public educational performances in the southeast. Upon presentation, the snake had a 1-cm by 1.5-cm subcutaneous, longitudinally ovoid swelling overlying his right ventral mandible area (Fig. 1A). He also had a 1-cm swelling overlying his right eye and extending down into the orbit, displacing the eyeball laterally and displacing the palate and dorsal limit of the choana ventrally. The masses were lobular, whitish in appearance, and enclosed in a thin capsule. The submandibular mass was removed in its entirety, as its capsule was very discrete. The other mass was very friable and locally extensive. Both masses were surgically removed and submitted for histopathological examination and culture. Not all portions of the second mass could be completely removed, due to the location of this mass, but the area enclosing it was debrided. At the time of surgery, the snake was treated with meloxicam (Metacam; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) at a dose of 0.2 mg/kg of body weight once a day and enrofloxacin (Baytril; Bayer HealthCare, LLC, Animal Health Division, Shawnee Mission, KS) at a dose of 5 mg/kg twice a day. This was continued until the histopathology report indicating a fungal infection was received. Enrofloxacin was discontinued, and ketoconazole was initiated. A single oral administration of ketoconazole (Apotex, Inc., Toronto, Ontario, Canada) at 50 mg/kg was administered daily. The snake was kept at 29.5°C and was tube fed Hill's a/d prescription diet (Hill's Pet Nutrition, Inc., Topeka, KS) A/D at 25 ml every 3 days. There was a moderate amount of postoperative swelling at the incision over the orbit. This was treated with warm wet compresses daily, and the swelling decreased. The snake passed away 2 months after surgery.

The histopathological evaluation and primary culture were performed at the University of Georgia, Veterinary Diagnostic and Investigational Laboratory, Tifton, GA. Both masses consisted of multifocal-to-coalescing granulomas. The granulomas had central regions of amorphous eosinophilic and occasional cellular debris, surrounded by an inflammatory cell infiltrate consisting of histiocytes, lymphocytes, and occasional heterophils (Fig. 1B). Mild concentric fibrosis surrounding these areas was observed. Moderate numbers of hyphae and closely segmented arthroconidiating hyphae were found primarily within the centers of the granulomas. The hyphae were 3 to 7 μm broad, parallel walled, segmented, and occasionally branching. Similar fungal structures were also observed with the use of a Grocott-Gomori methenamine silver stain (Fig. 1C).

Routine bacterial and fungal cultures were performed with the tissue sample. For fungal culture, a portion of the sample was inoculated on Sabouraud dextrose agar (Remel, Lenexa, KS) and incubated at 29°C for 4 weeks. Bacterial cultures were negative. A moderate-to-heavy and pure growth of a fungus was observed on fungal medium. Colonies were white and had sterile septate hyphae, and no fruiting bodies were present. The fungus was unidentifiable by conventional laboratory techniques. The isolate was forwarded to the Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio, San Antonio, TX, for morphological iden-

* Corresponding author. Mailing address: Veterinary Diagnostic and Investigational Laboratory, College of Veterinary Medicine, University of Georgia, 43 Brighton Road, Tifton, GA 31793. Phone: (229) 386-3340. Fax: (229) 386-7128. E-mail: srajeev@uga.edu.

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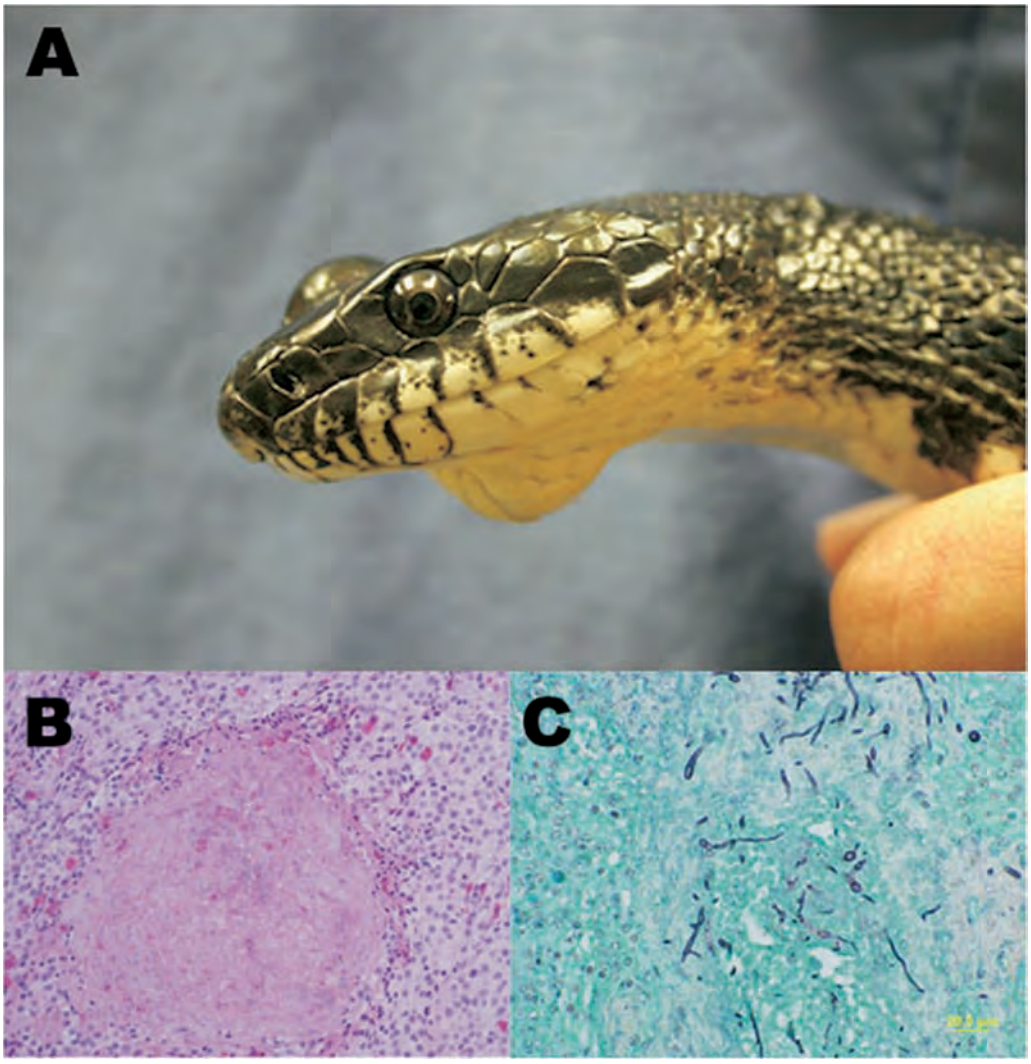

FIG. 1. (A) Cutaneous masses; (B) hematoxylin and eosin-stained section of the lesion; (C) Grocott-Gomori methenamine silver-stained section of the lesion.

tification and accessioned into the culture collection as UTHSC 07-604.

On potato flake agar (prepared in-house) (10) at 23°C, the colonies were white to pale yellow, with a similarly colored reverse side; were velvety to granular with age; resembled *Chrysosporium* colonies microscopically; displayed conidia borne on stalks as well as arthroconidia; and produced a strong, pungent odor. As the isolate did not appear to morphologically match any known *Chrysosporium* species, it was submitted to the Department of Microbiology and Immunology for molecular characterization under accession number R-3923.

The internal transcribed spacer (ITS) and D1-D2 regions were amplified using a DNA preparation methodology, primers, and PCR conditions as previously described (5, 12). PCR products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) and then sequenced at the Advanced Nucleic Core Facility of the University of Texas. Each sequence was then used to search GenBank, using the BLASTn algorithm at http://www.ncbi.nlm.nih.gov/. Sequencing of the ITS (614 bp in length; GenBank accession no. EU715819) and D1-D2 (486 bp in length; GenBank accession no. EU715820) regions failed to provide an unequivocal identification, as the closest D1-D2 maximum identity was 93% (*Onygena corvina*; GenBank accession no. AB075355) and the closest ITS maximum identity was 84% (*Arthroderma multifidum*; GenBank accession no. AB361651). However, sequence data confirmed the association of the clinical isolate with the onygenalean fungi. As the percentages of similarity with all the sequences deposited in GenBank were very low, a conclusive identity could not be made. The isolate was forwarded to the Mycology Unit at Rovira i Virgili University in Reus, Spain, where further extensive morphological and molecular phylogenetic studies were undertaken to characterize this fungus.

The morphological description of the present isolate is as follows. Colonies on potato carrot agar (PCA; 20 g potato, 20 g

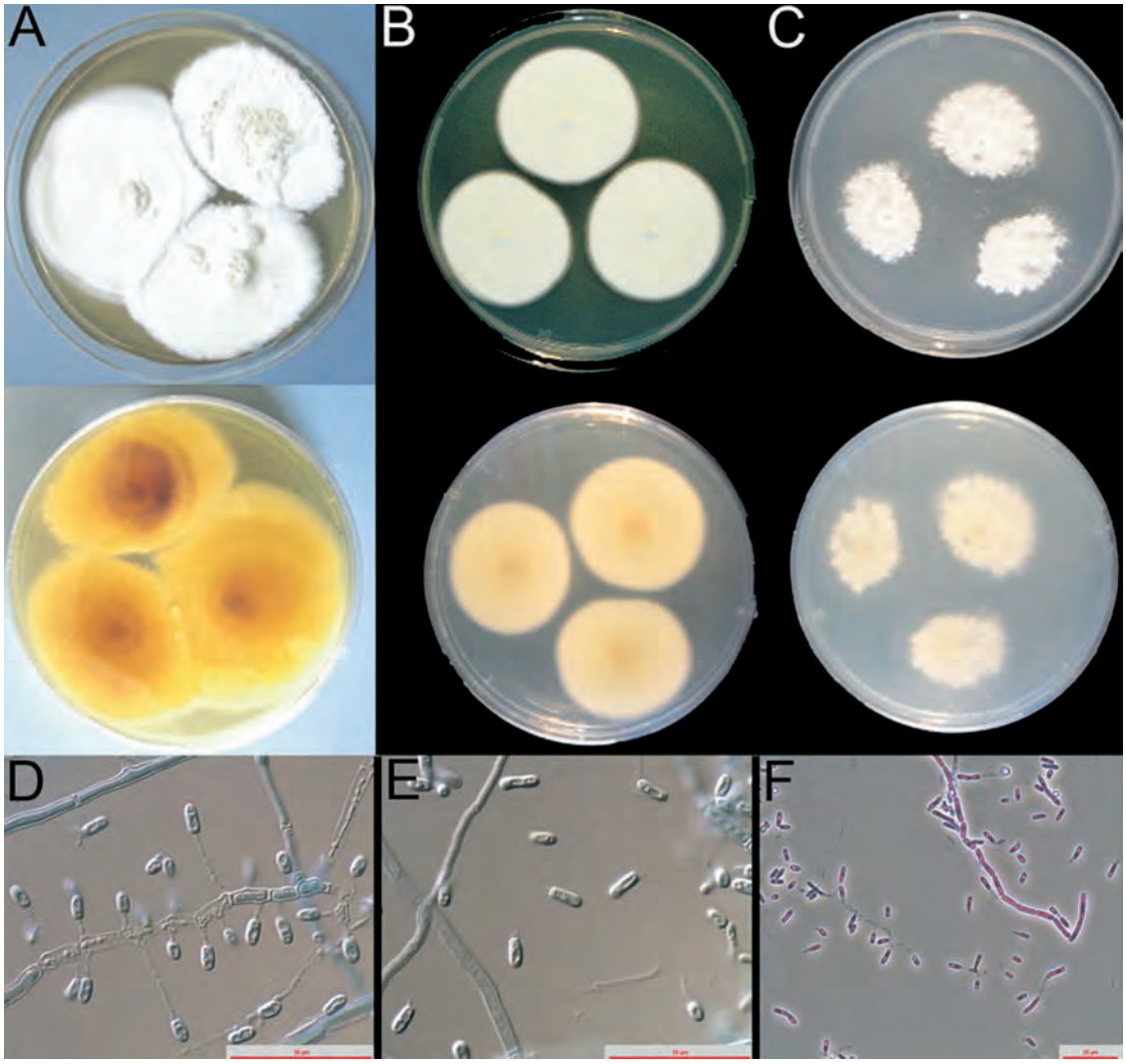

FIG. 2. Colonial and microscopic morphology of *Chrysosporium ophiodiicola* R-3923. (A) PYE, front and reverse; (B) potato dextrose agar, front and reverse; (C) PCA, front and reverse; (D) fertile hyphae and conidia; (E) conidia showing remnants of wall following rhexolytic dehiscence; (F) fertile hyphae with arthroconidia and terminal and lateral conidia.

carrot, 15 g agar, 1 liter water) (Fig. 2C) attained 27- to 29-mm diameters in 14 days at 25°C and were white, with an uncolored reverse side. They were felty, plane, and fimbriate, with a poorly defined margin. Sparse tufts of aerial mycelium were present on the submarginal zone. Vegetative hyphae were hyaline, branched, septate, smooth, and thin walled. They were 1.5 to 2.5 μm wide and often disarticulated at maturity to form cylindrical, 7.5- to 10- by 2- to 2.5 (3)-μm arthroconidia adjacent to each other. Fertile hyphae arise as lateral branches. Terminal and lateral conidia were borne on straight or flexuous side branches of variable length (4.5 to 16 μm) or were sometimes sessile. Conidia were unicellular, solitary, thin walled, smooth, hyaline to pale yellow, and cylindrical to slightly clavate (4.0 to 6.5 (9) by 2.0 to 3.0 μm) and were released by rhexolytic dehiscence, with broad and long basal scarring (Fig. 2D to F). Intercalary, solitary conidia were often present, similar to the terminal and lateral ones. Racquet hyphae were scarce, and chlamydospores were not observed. On potato dextrose agar (Difco Laboratories, Detroit, MI), the fungus grew more quickly and produced denser colonies, 31 to 35 mm in diameter, in 14 days at 25°C (Fig. 2B). They were white to pale yellow, buff after 1 month, and powdery, with droplets of colorless or light yellow exudates at the periphery. On phytone-yeast extract agar (PYE; BBL, Cockeysville, MD), the colonies had 32- to 39-mm diameters in 14 days at 25°C (Fig. 2A), and they were white and light yellow at the center, powdery, and dense, with the presence of droplets of colorless exudate at the center and a light brown reverse side. On oatmeal agar (30 g oat flakes, 1 g $MgSO_4 \cdot 7H_2O$, 1.5 g KH_2PO_4, 15 g agar, 1 liter tap water), the colonies were similar to those on PCA. The fungus had a very restricted growth at 15°C (5-mm diameter in 14 days). At 37°C, there was no growth. The colonies produced a strong, pungent (skunklike) odor after 1 month of incubation in all the media tested. Attempts to induce the teleomorph on oatmeal agar and sterile garden soil to which horse hair had been added were unsuccessful after 2

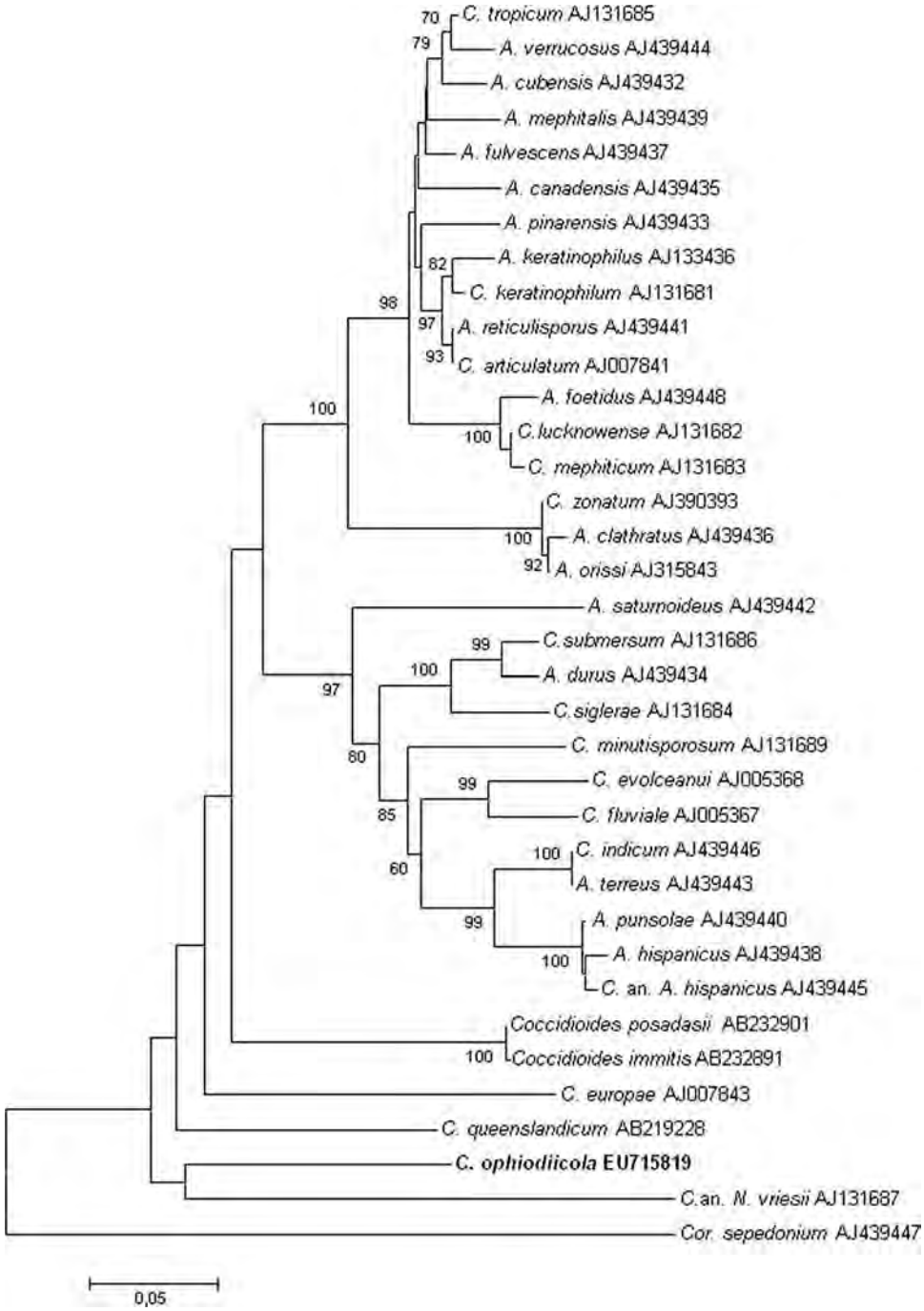

FIG. 3. Neighbor-joining tree based on Kimura two-parameter corrected nucleotide distances among ITS1-5.8s. ITS2 ribosomal DNA sequences of the species are compared with those of *Chrysosporium ophiodiicola*. Branch lengths are proportional to distance. Bootstrap replication frequencies over 70% (1,000 replications) are indicated on the nodes. Abbreviations: *A.*, *Aphanoascus*; *C.*, *Chrysosporium*; *Cor.*, *Corynascus*; *N.*, *Nannizziopsis*; an., anamorph.

months of incubation at 25°C. However, a strong keratinolytic activity was noticed.

The main characteristics of the snake isolate were the presence of numerous narrow, cylindrical-to-slightly clavate conidia and the strong, pungent odor of the colonies. This odor is not rare in the Onygenales, since strains of other species, such as *Chrysosporium mephiticum* Sigler and *Aphanoascus mephitalis* (Malloch & Cain) Cano & Guarro, show similar characteristics (12). However, these species can be easily differentiated from the present fungus by their morphology; *C. mephiticum* has pyriform-to-subglobose conidia occurring more or less synchronously, and *A. mephitalis* usually produces the teleomorph in culture and has a *Malbranchea* anamorph. In addition, these species show very different ITS sequences (4, 6,

14) (Fig. 3). Narrow cylindrical conidia are also produced by *Chrysosporium europae* Sigler, Guarro & Punsola. But this species can be easily differentiated from the new species by its characteristic vinaceous-buff-pigmented colonies on PYE and the absence of a strong, pungent odor (11).

The combined morphological, cultural, and molecular characteristics of the snake isolate do not correspond to any of the species within the genus *Chrysosporium* described to date. Thus, the following new species is proposed.

Chrysosporium ophiodiicola Guarro, D. A. Sutton, Wickes, and Rajeev, sp. nov. Etymology: from the Greek ophio, snake. Ad fungos conidiales, hyphomycetes pertinens. Coloniae in agaro cum decocto tuberorum et carotarum post 14 dies ad 25°C, 27- ad 29-mm diametro celeriter crescentes, planae, albae; reversum hyalinae. Coloniae in agaro cum decocto tuberorum post 14 dies ad 25°C, 31- ad 35-mm diametro; in agaro phytone extracto levedinis post 14 dies at 25°C, 32- ad 39-mm diametro. Ad 37°C incrementum nullum. Odor foetidus. Hyphae hyalinae vel subhyalinae, leviter ramosae, septatae, 1.5- ad 2.5-μm latae. Conidia terminalia et lateralia sessilia vel in ramae laterales, cylindrica vel clavatae, hyalina vel lutea, leviatunicata, 4.0 ad 6.5 (9) per 2.0 ad 3.0 μm; arthroconidia hyalina vel lutea, leviatunicata, cylindrica, 7.5 ad 10 per 2 ad 2.5 (3) μm. Chlamydosporae absunt. Teleomorphosis ignota. Species keratinolytica cultura typica: ex ophio pelle. In collectione fungorum CBS 122913 deposita est. Isotypus FMR 9510, UTHSC 07-604.

Phylogenetic analysis of the ITS region of *C. ophiodiicola* and other related onygenalean fungi was performed with MEGA 2.1 software (7), using the neighbor-joining method and basing the analysis on Kimura's two-parameter corrected nucleotide distances. The *Chrysosporium* anamorph of *Nannizziopsis vriesii* was the species nearest to *C. ophiodiicola* in the ITS neighbor-joining tree (Fig. 3). Both species are associated with infections in reptiles.

Chrysosporium ophiodiicola was isolated from a subcutaneous granuloma of a snake, which is not an unusual source for recovering chrysosporia. The *Chrysosporium* anamorph of *Nannizziopsis vriesii* has been isolated from cases of dermatitis in snakes (2, 15), chameleons (9), crocodiles (13), and bearded dragons (3) and from a nasal granuloma in an Ameiva lizard (8). In a recent report, a *Chrysosporium* species related to *Nannizziopsis vriesii* was isolated from a case of cutaneous hyalohyphomycosis from two green iguanas (1). Phenotypically, *C. ophiodiicola* can be separated from the *Chrysosporium* anamorph of *Nannizziopsis vriesii* by the absence of asperulate fertile hyphae and globose-to-pyriform conidia sometimes grouped in clusters and the presence of an odor in the colonies of the former.

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Disseminated phaeohyphomycosis in weedy seadragons (*Phyllopteryx taeniolatus*) and leafy seadragons (*Phycodurus eques*) caused by species of *Exophiala*, including a novel species

Akinyi Nyaoke, E. Scott Weber, Charles Innis, Donald Stremme, Cynthia Dowd, Lynn Hinckley, Timothy Gorton, Brian Wickes, Deanna Sutton, Sybren de Hoog, Salvatore Frasca Jr.[1]

Abstract. During the period from January 2002 to March 2007, infections by melanized fungi were identified with greater frequency in aquarium-maintained leafy seadragons (*Phycodurus eques*) and weedy seadragons (*Phyllopteryx taeniolatus*), pivotal species to the educational and environmental concerns of the aquarium industry and conservation groups. The objective of this study was to characterize the pathology and identify fungi associated with phaeohyphomycotic lesions in these species. Samples from 14 weedy and 6 leafy seadragons were received from 2 institutions and included fresh, frozen, and formalin-fixed tissues from necropsy and biopsy specimens. Fresh and frozen tissues were cultured for fungi on Sabouraud dextrose agar only or both Sabouraud dextrose agar and inhibitory mold agar with gentamicin and chloramphenicol at 30°C. Isolates were processed for morphologic identification and molecular sequence analysis of the internal transcribed spacer region and D1/D2 domains of the large subunit ribosomal RNA gene. Lesions were extensive and consisted of parenchymal and vascular necrosis with fungal invasion of gill (11/20), kidney (14/20), and other coelomic viscera with or without cutaneous ulceration (13/20). *Exophiala* sp. isolates were obtained from 4 weedy and 3 leafy seadragons and were identified to species level in 6 of 7 instances, namely *Exophiala angulospora* (1) and a novel species of *Exophiala* (5), based on nucleotide sequence comparisons and phylogenetic analyses. Disseminated phaeohyphomycosis represents an important pathologic condition of both weedy and leafy seadragons for which 2 species of *Exophiala*, 1 a novel species, have been isolated.

Key words: *Exophiala*; phaeohyphomycosis; *Phycodurus eques*; *Phyllopteryx taeniolatus*; seadragons.

Introduction

The term ''phaeohyphomycosis'' is collectively used for cutaneous, subcutaneous, and systemic diseases caused by several genera of septate dark-walled fungi, referred to as ''dematiaceous,'' ''phaeoid,'' or ''melanized.'' Melanized fungi exhibit a high degree of molecular diversity with more than 100 species in 60 genera.[40] These agents, classified in various orders of the fungal kingdom, are ubiquitous and primarily recognized as soil saprophytes, plant pathogens, and environmental contaminants. Melanized fungi have been associated with disease in humans,[6,57] mammals,[11,18,28,61] birds,[32,56] amphibians,[12] reptiles,[30] fish,[19,60] and invertebrates.[8,62]

Of special interest are members of the order *Chaetothyriales*, such as *Exophiala* and *Cladophialophora*, which are ecologically different and seem to be associated with assimilation of alkylbenzenes, compounds that are also present in vertebrate bodies.[50] These fungi are regularly encountered as causative agents of mycoses of medical and veterinary importance; only *Onygenales*, the order containing dermatophytes and dimorphic pathogens, has a comparable number of clinically relevant fungi.[16,17]

Species of *Exophiala* represent a source of emerging fungal cutaneous, subcutaneous, and systemic infections, especially in immunocompromised human patients,[9,36,52,53,68] and animals.[10,27,31,39] In teleostean and cartilaginous fishes, *Exophiala* sp. infection has been reported in Atlantic salmon (*Salmo salar* L.)[46,54] and cutthroat trout (*Oncorhynchus clarkii*),[13] caused by *Exophiala salmonis*, in channel catfish (*Ictalurus punctatus*)[42] and smooth dogfish (*Mustelus canis*)[23] due to *Exophiala pisciphila*, and in captured King George whiting (*Sillaginodes punctata*)[51] in associa-

From the Connecticut Veterinary Medical Diagnostic Laboratory, Department of Pathobiology and Veterinary Science (Nyaoke, Hinckley, Frasca) and Center of Excellence for Vaccine Research (Gorton), University of Connecticut, Storrs, CT; New England Aquarium, Boston, MA (Weber, Innis); Adventure Aquarium, Camden, NJ (Stremme, Dowd); Department of Microbiology (Wickes) and Fungus Testing Laboratory, Department of Pathology (Sutton), The University of Texas Health Science Center at San Antonio, San Antonio, TX; and Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (de Hoog).

[1] Corresponding Author: Salvatore Frasca, Jr., Department of Pathobiology and Veterinary Science, U-3089, University of Connecticut, 61 North Eagleville Road, Storrs, CT 06269. salvatore.frasca@uconn.edu

tion with an *Exophiala* sp. Most recently, a novel species of *Exophiala* was isolated from Japanese flounder (*Paralichthys olivaceus*).[33]

Seadragons are marine fish of the family *Syngnathidae*, which include the fused jawed fishes, such as seahorses and pipefish. Seadragons inhabit the shallow, temperate waters along the southern and western Australian coastline and Tasmania and are listed as near threatened on the Red List of Threatened Species by the International Union for the Conservation of Nature and Natural Resources (www.iucn.org). There are 2 main genera with a single species in each genus, namely the weedy seadragon (*Phyllopteryx taeniolatus*) and the leafy seadragon (*Phycodurus eques*). Seadragons are significant exhibit species of the aquarium industry and conservation groups because of their importance to research and educational efforts focused on marine coastal habitat conservation and ecosystem sustainability (www.dragonsearch.asn.au).[14,15] Over a period of 5 years from January 2002 to March 2007, slightly more than 400 seahorses, pipefish, and seadragons from several zoos and approximately a dozen commercial aquariums have been submitted to the Connecticut Veterinary Medical Diagnostic Laboratory (CVMDL; University of Connecticut, Storrs, CT) for necropsy. Of these syngnathid submissions, infections with melanized fungi were prevalent in leafy and weedy seadragons from 2 different aquariums. This report describes the characteristics of disseminated phaeohyphomycosis in seadragons caused by *Exophiala* spp., including infection by a novel (i.e., as yet undescribed) species referred to as "*Exophiala* sp. nov."

Material and methods

Animals

Specimens consisted of juvenile and adult, captive-hatched seadragons reared in Australia prior to legal importation into the United States. Gender was undetermined for most animals due to sexual monomorphism when not in breeding condition. Animals were housed in groups of 1–10 animals in both species-specific and mixed species groups. Animals were maintained in filtered, natural, or artificial sea water at temperatures of 14–16°C, pH 8.0–8.2, total ammonia nitrogen <0.07 mg/l, nitrite 0 mg/l, nitrate <30 mg/l, and salinity 29–36 g/l in tanks ranging from 1,000 to 8,000 liters. Life support systems included mechanical and biological filtration with protein skimmers and ultraviolet and ozone disinfection. Diet consisted of live and frozen mysis shrimp (*Mysidacea* sp.), frozen zooplankton, and live brine shrimp (*Artemia salina*).

Weedy and leafy seadragons were submitted as part of routine diagnostic investigations to the CVMDL between January 2002 and March 2007. Fish were submitted from 2 different commercial aquariums located in 2 U.S. states and were presented either live or fixed in formalin. Seadragons were euthanized using an approximate dose of 400 mg/l of tricaine methanesulfonate[a] and were observed for 15–30 min past the last active opercular movement in consideration of the guidelines provided by the American Veterinary Medical Association[3] at the CVMDL or at the submitting institutions, or the animals died naturally. After initial identification of *Exophiala* sp., some animals were treated with little success using a variety of topical and systemic antifungal agents, including fluconazole,[b] voriconazole,[b] itraconazole,[c] terbenifine,[d] 37% w/v formaldehyde solution,[d] methylene blue,[d] malachite green,[e] acriflavine,[e] and Virkon.[f] Therapy for concurrent bacterial and protozoal infections varied for each case and included at least 1 of the following drugs: ceftazidime,[g] oxytetracycline, triple sulfa powder, metronidazole, kanamycin sulfate powder,[e] and chloroquine.[h]

Necropsy and histopathology

Gross necropsies were performed within 12 hr of death. Representative tissue samples or swabs of lesions were aseptically collected for wet mount preparations and microbial culture. Animals were dissected into multiple tissue samples or had gills and coelomic viscera exposed by removal of the operculums and a ventral midline incision with or without removal of the lateral body wall. Tissue samples or partially opened whole specimens were then fixed by immersion in 10% neutral buffered formalin. Bony tissues were decalcified after fixation using 0.5 M (molar [solution]) ethylenediamine tetra-acetic acid for 24–36 hr prior to trimming.

For preparation of histologic sections, formalin-fixed tissue samples were trimmed to fit plastic cassettes, routinely processed, embedded in paraffin, sectioned at 4 μm, mounted on glass slides, stained with hematoxylin and eosin, and then examined by bright field microscopy. Additional sections were stained with Fontana-Masson (FM), periodic acid–Schiff (modified McManus stain), and Grocott's methenamine silver techniques to highlight histomorphologic and staining characteristics of the fungi.[5,59]

Microbial culture and fungus identification

Tissue samples and swabs were submitted for microbial testing, which included aerobic bacterial culture and separate fungal culture. Bacterial cultures from 4 weedy seadragons and 1 leafy seadragon were performed at a commercial veterinary diagnostic laboratory (IDEXX Laboratories, North Grafton, MA). Fungal cultures were performed at the CVMDL, where tissue samples were seared, sliced with a sterile surgical blade, and sampled with a cotton-tipped swab, which was then used to streak plates of Sabouraud dextrose agar[i] and inhibitory mold agar with gentamicin and chloramphenicol[j] in duplicate; plates were sealed using parafilm and incubated at 30°C. Swabs submitted for fungal culture were used directly to streak duplicate plates of the 2 types of media, which were then incubated at 30°C. Plates were incubated for up to 4 weeks and examined weekly for growth of mold. In cases in which

an olivaceous to black, velvety mold was isolated, 1 plate of the pair was transferred to the Fungus Testing Laboratory (The University of Texas Health Science Center at San Antonio [UTHSCSA], San Antonio, TX) for morphologic and molecular identification.

Isolates were then transferred onto potato flakes agar (PFA) plates prepared in house and incubated at 25°C.[55] Both the macroscopic morphology of the colonies and the diagnostic microscopic features were determined from this medium after approximately 12 days of incubation. Microscopic features were studied using the slide culture technique[26] and modified to contain PFA rather than water agar for both the nutrient and the moisture source. Temperature studies were performed at 30°C, 35°C, and 40°C on PFA slants; nitrate assimilation was assessed using previously described methods.[49] Brown pigment formation was evaluated on Sabouraud dextrose agar.

Molecular fungus identification and phylogenetic analysis

Genomic DNA was extracted from conidia recovered from a 72-hr PFA plate using Prepman Ultra reagent[k] according to the manufacturer's instructions. Two polymerase chain reaction (PCR) amplifications were performed on each isolate, and molecular analyses were focused on ribosomal genes for phylogenetic inference. The first reaction amplified the internal transcribed spacer (ITS) region located between the 18S and 28S rRNA genes using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′).[21,65] The second reaction amplified the D1/D2 region of the 28S rRNA gene using primers NL-1 (5′-GCATATCAA-TAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCC-GTGTTTCAAGACGG-3′).[34,48] Both PCR amplifications were performed in 50-µl reaction volumes using Triple Master Taq DNA polymerase,[d] each deoxyribonucleotide triphosphate (dNTP),[l] and primers (prepared at the UTHSCSA Nuclear Core Facility) at concentrations specified by the manufacturer's instructions. All PCR amplifications were performed in a commercial thermocycler[m] using a preprogrammed 3-step protocol as the standard program for all reactions. Cycling conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 30 cycles, each cycle consisting of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. Amplification success was confirmed by agarose gel electrophoresis. The remaining template DNA was then cleaned by polyethylene glycol precipitation.[l] Sequencing was performed on both strands using the PCR primers as sequencing primers at the UTHSCSA Advanced Nucleic Acids Core Facility. The resultant sequences were compared with available sequences at the National Center for Biotechnology Information using BLASTn (nucleotide database using a nucleotide query, http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) searches of the databases,[1,2] as well as by comparison to the sequence database of black molds at the Centraalbureau voor Schimmelcultures (CBS; http://www.cbs.knaw.nl/databases/). The ITS sequences were aligned using BioNumerics 4.6 software.[n] A substitution model was calculated using MrAIC (http://www.abc.se/~nylander), and the tree was built using TREEFINDER algorithm (http://www.treefinder.de) version June 2007 and bootstrapping with 1,000 replicates; values >80 are shown with the branches.

Results

Animals

Twenty aquarium-held weedy (14) and leafy (6) seadragons were evaluated. Animals were in the collection for 2 weeks to 4 years (mean = 32 weeks) prior to onset of clinical signs. Duration of clinical signs ranged from 1 week to 6 months (mean = 8 weeks). Clinical signs included weakness, loss of appetite, lethargy, increased respiratory rate and effort, abnormal buoyancy, listing, piping at the surface of the water, and death. Fungal dermatitis was diagnosed antemortem in 6 cases via cytology or biopsy of lesions, and antemortem fungal culture isolated *Exophiala* sp. nov. in 2 such cases.

Pathologic findings

Seadragons were submitted to the CVMDL with either antemortem evidence of fungal infection from cytology or biopsy or black lesions suggestive of phaeohyphomycosis. Lesions were identified in skeletal muscle (18/20), skin (16/20), kidney (14/20), gill (11/20), swim bladder (7/20), heart (2/20), liver (3/20), spleen (1/20), muscle coats and serosa of the intestine (2/20), mesentery (1/20), and extradural sinus and spinal cord (6/20); 15 seadragons had lesions in 3 or more of these tissues (Table 1). The most obvious gross lesions were identified in the skin and consisted of 1 to several well-demarcated, occasionally extensive, ulcerations, often with raised black margins, located randomly over the head, trunk, dorsum of the tail, at the base of fins, or in skin around the cloaca (Fig. 1). Microscopically, cutaneous lesions were characterized by ulcerations of the epidermis and extensive mats of fungal hyphae that invaded dermal fibrous connective tissue and extended into the underlying hypodermis, fascia, and skeletal muscle with myonecrosis and mild histiocytic infiltrates.

Multiple, well-demarcated, and occasionally extensive black foci were identified grossly in the kidney, gill, swim bladder, and intestinal wall of seadragons upon internal examination. Microscopically, renal lesions consisted of extensive regions of necrosis involving tubules, hematopoietic interstitium, and sinusoids that were infiltrated throughout by fungal hyphae and corresponded to grossly visible black, friable parenchyma (Fig. 2). Hyphae invaded the overlying epaxial muscle with myonecrosis and mild histiocytic infiltrates. Gill lesions consisted of focally extensive necrosis of filaments, their lamellae, the

Table 1. Anatomic distribution of phaeohyphomycotic lesions in seadragons in the current study.*

Animal ID/species	Gender	Aquarium	Gill	Skin	Skeletal muscle	Kidney	Swim bladder	Heart	Intestine	Mesentery	Liver	Spleen	Extradural sinus and spinal cord†
2002 #1 LSD	F	NEAq	X	X	X								
2003 #1 WSD	U	NEAq		X									
2003 #2 WSD	M	NEAq	X	X	X	X	X						
2003 #3 LSD	U	NEAq		X	X								
2003 #4 LSD	F	NEAq	X		X	X							X
2003 #5 WSD	U	NEAq		X	X	X							
2003 #6 LSD	F	NEAq			X	X	X						
2004 #1 WSD	F	AAq	X	X	X	X	X			X	X	X	
2004 #2 WSD	U	AAq		X	X	X	X						X†
2004 #3 LSD	U	NEAq	X	X	X	X			X		X		
2004 #4 WSD	U	AAq		X	X								
2005 #1 WSD	U	NEAq	X			X							
2005 #2 WSD	U	NEAq	X	X	X	X							
2005 #3 WSD	F	AAq	X		X	X	X				X		
2005 #4 WSD	U	NEAq	X	X	X	X		X					
2005 #5 WSD	U	NEAq		X	X	X	X	X	X				X†
2006 #1 WSD	M	AAq	X	X	X	X							X
2006 #2 WSD	U	NEAq	X	X	X	X	X						X†
2006 #3 WSD	F	NEAq		X	X								X
2007 #1 LSD	F	NEAq		X	X								
Total	20		11	16	18	14	7	2	2	1	3	1	6

* LSD = leafy seadragon (*Phycodurus eques*); WSD = weedy seadragon (*Phyllopteryx taeniolatus*); M = male; F = female; U = undetermined; NEAq = New England Aquarium, Boston, MA; AAq = Adventure Aquarium, Camden, NJ.

† Fungal hyphae were present in the spinal cord.

underlying connective tissue, and blood vessels of the gill arch, with minimal to occasionally moderate loosely organized infiltrates of macrophages (Fig. 3). Lesions in the intestine and swim bladder were limited to the muscle coats and serosa and were characterized by foci of myonecrosis, infiltrated by fungal hyphae, and minimal to moderate, loosely organized infiltrates of macrophages. In the liver, hyphae invaded venules and sinusoids with dissociation and necrosis of hepatocytes and pancreatic acini in 2 instances. Hyphae coursed through venules of the liver and the reticuloendothelial stroma of the spleen and invaded mesenteric blood vessels in 1 specimen. Small numbers of hyphae were present in the extradural sinus of 6 specimens and invaded the spinal cord in 3 of these instances.

Lumina of blood vessels of gill and viscera contained intertwined fungal hyphae together with variable amounts of fibrin, serum protein, and necrotic leukocytes, accompanied by necrosis of vessel walls (Fig. 4). Fungal hyphae were 2–3 μm in width, slender, filamentous, and septate, with right-angle branching and thin parallel walls that stained brown in routine hematoxylin and eosin–stained sections and in sections prepared using the Fontana-Masson technique, consistent with the expected histochemical staining reaction of melanized fungi (Fig. 5).[66,67] Ciliated protozoa, consistent with *Uronema* sp., and aggregates of Gram-negative, rod-shaped bacteria were identified on cytology of affected gill and skin in 5 cases. Other pathologic findings included enteric coccidiosis in weedy seadragons (2/20), biliary (1/20) and renal myxozoanosis (5/20) in weedy seadragons, and parasitic (3/20) and mycobacterial (1/20) granulomas in visceral organs.

Microbial culture and fungus identification

Fungal cultures were attempted in 4 weedy and 3 leafy seadragons involving 1 or more samples from kidney, skin, liver, and/or spleen; isolates were identified to species level in 6 of 7 instances (Table 2). Isolates grew on both Sabouraud dextrose agar and inhibitory mold agar with gentamicin and chloramphenicol at 30°C over a period of 1–3 weeks. Isolates yielded velvety, olivaceous, or brown-black molds and were referred to the Fungus Testing Laboratory over several months. In most isolates, the colonies were generally restricted, were somewhat moist initially, had a moderate growth rate, were olivaceous to olivaceous-gray with a black reverse, and became velvety at maturity. Both *E. angulospora* and *Exophiala* sp. nov. were initially yeast-like but displayed budding cells throughout their growth cycle. These cells soon germinated with the formation of pale, olivaceous torulose hyphae. Conidiation was annellidic, and annelloconidia were either single-celled (2–3 μm × 4–5 μm for *Exophiala* sp. nov.; Fig. 6) or commonly angular (2–3 μm × 4–6 μm for

E. angulospora). Conidia were borne in slimy masses at the tips of flask-shaped to cylindrical annellides and from intercalary conidiogenous loci. All isolates assimilated nitrate, none were able to grow at 35°C, and none produced a brown diffusible pigment on Sabouraud dextrose agar suggestive of *E. dermatitidis*. Given the very similar microscopic and physiologic profiles of several species of *Exophiala*, only *E. angulospora*, which produces distinct angular annelloconidia, could be identified with reasonable certainty based upon morphologic features alone. A variety of Gram-negative bacteria, including *Vibrio* sp. and *Pseudomonas* sp., were cultured from lesions. No species of bacteria was consistently isolated from these lesions.

Molecular fungus identification and phylogenetic analysis

The ITS tree was built using TREEFINDER with substitution model GTR+G according to MrAIC calculations. The same program calculated bootstrapping values that gave 100% for all branches. Isolates were found to belong to distinct species of *Exophiala*. In addition to *E. angulospora*, a second, hitherto unnamed species was encountered, clearly separate from any other taxon based on rDNA ITS sequences. Because many species of black yeasts differ by mutations rather than by indels or amplicon lengths, greater than 1% ITS sequence diversity exceeds the species level in this group. Figure 7 gives an overview of clades of nearest taxa in the order *Chaetothyriales*. Sequences dH16401, dH13448, and CBS 119918 represent isolates obtained from 1 leafy seadragon sampled in 2000 by 1 of the 2 aquariums. These sequences were initially identified as *E. pisciphila* but were subsequently found to be *Exophiala* sp. nov. when submitted to the CBS for molecular characterization along with sequences from other *Exophiala* sp. isolates obtained from seadragons and other aquatic animals. Tissue samples for histopathologic evaluation were not received from this 2000 seadragon for inclusion in the current study; nevertheless, inclusion of these sequences together with sequences of *Exophiala* isolates from other fish contributes to the distinct clade designation and host predilections that characterize *Exophiala* sp. nov. Nearly all species belonged to the black yeast genus *Exophiala* having annellidic conidiogenesis, whereas *Veronaea botryosa* with large sympodial conidiophores was found to be a member of this clade. Most species in the clade have originated from watery environments and have also been isolated from diseased fish and amphibians. In some species, strains from human origins were present and mostly associated with mild cutaneous infections. Efforts are presently under way to provide a formal description of *Exophiala* sp. nov. in a taxonomic paper, wherein this species will be introduced as a novel taxon (M. J. Harrak, G. S. de Hoog, unpublished data).

Discussion

Environmental fungi are increasingly important sources of infection to humans and animals. The emergence or resurgence of fungi as pathogens, including those previously considered environmental contaminants, have been associated with a wide range of globally relevant medical, societal, and economic factors, such as increasing populations of immunocompromised individuals,[4,22,36,52,53] international travel, changes in land use and agriculture, and even migration of clouds of desert dust in the atmosphere.[25] International commerce of farmed North American bullfrogs (*Rana catesbeiana*) used for the restaurant trade, for example, has been implicated as a cause of chytridiomycosis in wild amphibians elsewhere around the globe.[41] Ornamental fish and the aquarium industry represent a commercial source of regional and international translocation of innumerable species that can afford environmental fungi the opportunity to infect new hosts.

Exophiala spp. are ubiquitous in soil and aquatic environments and often considered environmental contaminants. Reports of *Exophiala* spp. infection in domestic and wild animals are few and include subcutaneous[10] and systemic[27] lesions in cats by *E. jeanselmei* and a subcutaneous mass in the right neck of a dog by *E. dermatitidis*.[31] In nondomestic animals, *E. jeanselmei* was isolated from a subcutaneous lesion in a free-ranging eastern box turtle,[30] and an *Exophiala* sp. with close similarity to *E. pisciphila* was isolated from systemic lesions in a Galapagos tortoise.[39] *Exophiala* spp., including *Wangiella (Exophiala) dermatitidis*, *E. jeanselmei*, *E. oligosperma*, and *E. spinifera*, are more common causes of phaeohyphomycotic lesions in immunocompromised humans and encompass ocular,[47] cutaneous, subcutaneous,[68] and occasional systemic infections, wherein a history of chronic debilitating disease, altered immune status, and/or chemotherapy was reported.[9,36,52,53] *Exophiala salmonis*, known to be a pathogen of fish,[13,46,54] has been associated with subcutaneous phaeohyphomycosis in a human patient who most likely acquired the infection from a water source.[38] This highlights the zoonotic potential of *Exophiala* spp. and the potential effect aquatic pathogens may have on human health.

Exophiala spp. have been identified as significant pathogens of cultured fish, such as cutthroat trout,[13] Atlantic salmon,[46] channel catfish,[42] and Japanese flounder,[33] resulting in localized and systemic infections with a notable variety of inflammatory

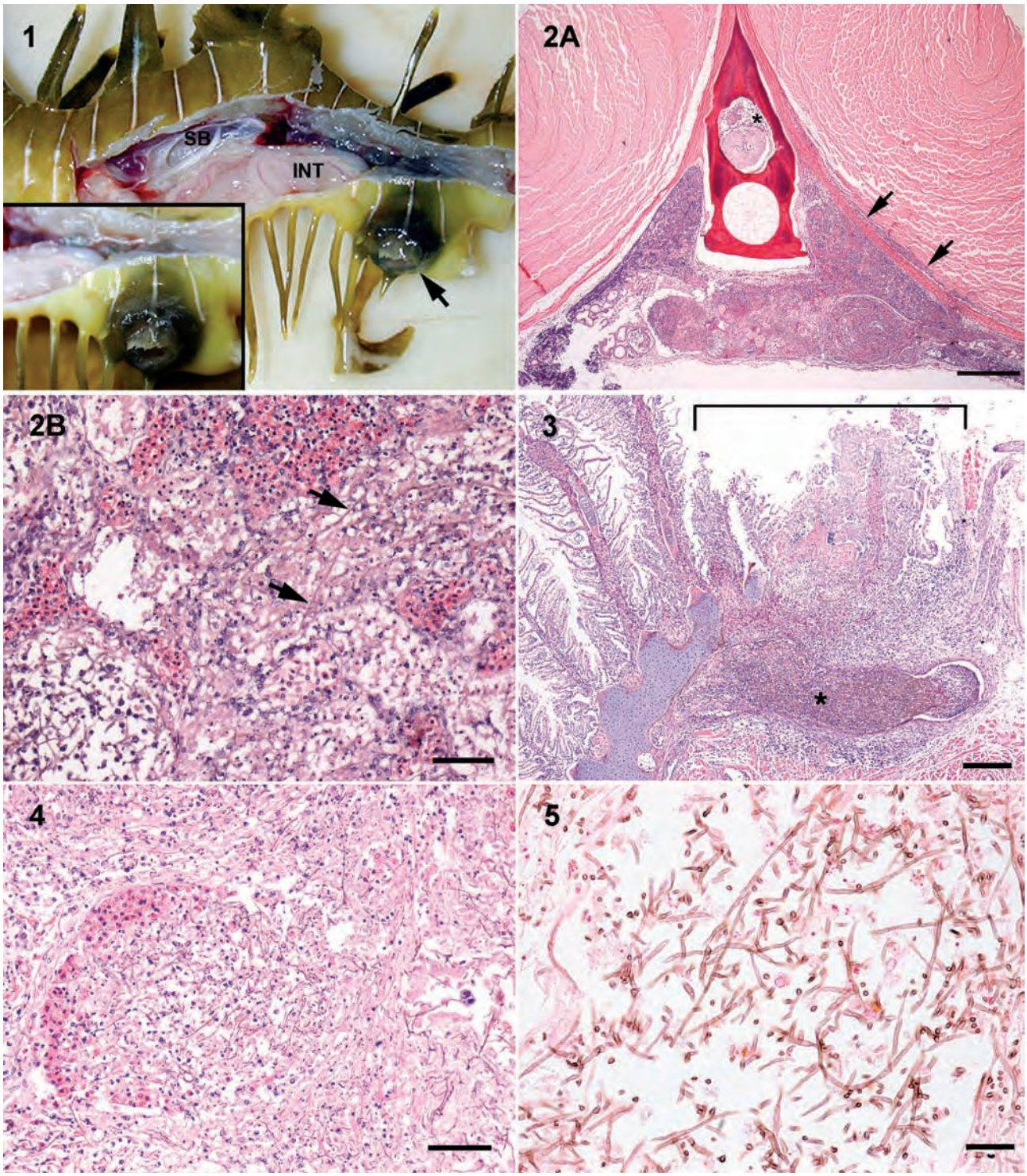

Figure 1. Skin ulcer; leafy seadragon with lateral body wall removed to expose coelomic viscera. An ulcer (arrow) is located in the skin adjacent to the cloaca. Inset: Closer view of the ulcer with raised black margins. SB = swim bladder; INT = intestine.

Figure 2. Transverse section of dorsal trunk; weedy seadragon. **A**, there is extensive necrosis involving approximately two-thirds of the renal parenchyma. Note the presence of fibrin and cells in the extradural sinus (asterisk) and an infiltrate along the fascia and margin of adjacent epaxial muscle (arrows). Hematoxylin and eosin. Bar = 500 μm. **B**, higher magnification of renal parenchyma reveals innumerable, filamentous brown fungal hyphae (arrows) coursing through necrotic tubules, interstitium, and sinusoids. Hematoxylin and eosin. Bar = 50 μm.

Figure 3. Gill; leafy seadragon. There is focally extensive necrosis of several consecutive filaments and their lamellae (bracket) overlying a region of the arch wherein a mat of densely intertwined brown fungal hyphae (asterisk) resides within the venous sinus.

Table 2. *Exophiala* sp. isolated from seadragons in the current study.*

Animal ID/Species	Aquarium	*Exophiala* sp. isolated
2002 #1 LSD	NEAq	*Exophiala* sp. nov.
2004 #3 LSD	NEAq	*Exophiala* sp. nov.
2005 #3 WSD	AAq	*Exophiala* sp. nov.
2005 #5 WSD	NEAq	*Exophiala angulospora*
2006 #1 WSD	AAq	*Exophiala* sp. nov.
2006 #3 WSD	NEAq	*Exophiala* sp. nov.

* Note the isolation of *Exophiala* sp. nov. from 2 different institutions (NEAq = New England Aquarium, Boston, MA; AAq = Adventure Aquarium, Camden, NJ). LSD = leafy seadragon (*Phycodurus eques*); WSD = weedy seadragon (*Phyllopteryx taeniolatus*).

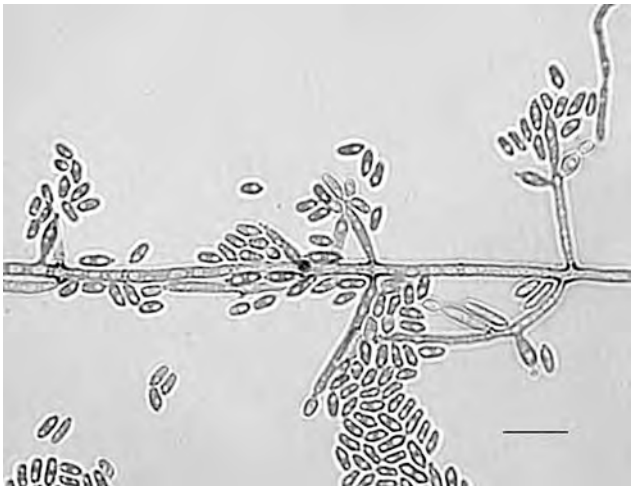

Figure 6. Microscopic colonial morphology of *Exophiala* sp. nov. showing septate hyphae with multiple annellides and conidiogenous loci bearing single-celled, approximately 2–3 μm × 4–5 μm conidia. Lactophenol cotton blue. Bar = 10 μm.

responses. A dematiaceous fungus subsequently named *Exophiala salmonis* was reported to be the cause of epizootics of cerebral mycetoma in fingerling cutthroat trout.[13] The lesion due to *E. salmonis* was characterized by granuloma formation with numerous giant cells in the brain and cranial tissues; it developed first in the brain, then extended peripherally to include surrounding cranial structures, such as the eye and gill. Similarly, in *Exophiala pisciphila* infection associated with high mortality of Atlantic salmon, hyphae invaded cranial structures, including semicircular canals, and the lateral line, accompanied by a granulomatous inflammatory reaction.[35] Contaminated food was suggested as a source of infection by *E. salmonis* in Atlantic salmon that developed granulomas in the posterior kidney.[54] *Exophiala salmonis* infection was later described in 3 Atlantic salmon held in a partial reuse system for up to 20 months.[46] Lesions in these salmon were systemic, including the kidney, and differed from earlier reports in that the host inflammatory response was predominantly granulocytic, with the formation of microabscesses. In contrast, lesions in Japanese flounder were limited to the skin.[33]

In the current study, systemic necrotizing lesions and invasion of blood vessels were consistent features of *Exophiala* spp. infection in seadragons. Necrosis was the predominant pathologic change, and the host inflammatory infiltrates were mild compared with the extent of necrosis and consisted mainly of histiocytes. Granulomas and abscesses were not consistently identified in seadragons, nor was there involvement of the brain, vestibular apparatus, or lateral line. Three of 20 seadragons did, however, have fungal hyphae that invaded the spinal cord, which may have resulted from invasion of the overlying extradural sinus.

The portal of entry in these cases is presumed to be gill or skin by traumatic inoculation or secondary inoculation of preexisting lesions, such as abrasions of the tubed snout, with subsequent hematogenous dissemination to visceral organs. Another portal of entry to consider could be direct ingestion of fungi associated with detritus and live or thawed frozen food accumulating in the tank substrate in aquarium settings. In the seadragon cases, however, there were no intestinal mucosal lesions associated with fungal invasion; instead, lesions were limited to the muscle coats and serosa, suggestive of hematogenous or transcoelomic spread.

The absence of granuloma formation or significant host inflammatory reaction to *Exophiala* spp. infection in these seadragons could be the result of an inadequate or deficient host immunologic response. No studies have been conducted to determine immune function in seadragons, but it could be that stress of captivity may be a contributory factor to reduced immune function. Fish in captive, artificial systems, or intensive rearing operations may be

←

Hematoxylin and eosin. Bar = 200 μm.

Figure 4. Blood vessel, kidney; weedy seadragon. Intertwined hyphae are present in the blood vessel lumen, and there is necrosis of a segment of the wall. Hematoxylin and eosin. Bar = 200 μm.

Figure 5. Fungal hyphae, kidney; weedy seadragon. Hyphae are slender, filamentous, and septate with occasional right-angle branches. Walls of hyphae stain brown, indicative of melanin. Fontana-Masson. Bar = 25 μm.

Figure 7. Consensus tree of prevalently waterborne *Exophiala* species, based on internal transcribed spacer (ITS) ribosomal DNA of 68 strains, constructed with neighbor-joining algorithm under the HKY+G substitution model (according to MrAIC), with 1,000 bootstrap replicates (according to TREEFINDER; values ≥80 are shown with the branches) and edited with Fig Tree version 1.0. Sequences are trimmed at GGCCC to (T/C)AGGGA for comparison. *Veronaea botryosa* was selected for rooting the tree. ITS sequences from *Exophiala* sp. nov. isolates form a distinct clade separate from other *Exophiala* species and supported by very high bootstrap value (100). Symbols following taxa (*, †, ‡) indicate sequences of isolates obtained from the same individual.

immunosuppressed or otherwise compromised and therefore predisposed to infections by *Exophiala* spp. and other environmental fungi.[46] Reports of infection with pigmented fungi in tropical marine fish are usually believed to be secondary to immunosuppression resulting from transport, trauma, or confinement-induced stress.[60] There were no mortalities of other species of fish housed in the same tanks as these seadragons, suggesting that this infection is species specific. This hypothesis is supported by the fact that species in the clade of psychotolerant, waterborne melanized fungi (*Chaetothyriales*) each show somewhat different host predilections (Fig. 7).

Studies on virulence factors in phaeoid fungi, such as *Wangiella (Exophiala) dermatitidis*, have identified synthesis of melanin within cell walls as the main virulence factor.[20,29,44] Melanin scavenges free radicals and hypochlorite produced by phagocytic cells and/or binds hydrolytic enzymes, and it is also believed to be important in the formation of the fungal appressorium, a structure that aids in entering the host cells.[20,29,63] The ability of these fungi to cause disseminated infection has been associated with the resistance afforded by melanin to oxidative damage by host phagocytic mechanisms. Melanin production in the context of host infection is not limited to phaeoid fungi, however, and melanin has been identified as a virulence factor in certain dimorphic fungi, such as *Cryptococcus neoformans*,[64] *Paracoccidioides brasiliensis*,[24] and *Histoplasma capsulatum*,[45] fungi that are considered emerging pathogens in both domestic animals and humans. Few studies have examined the role of chitin as a virulence factor in melanized fungi. Chitin serves to provide additional strength to fungal cell walls,[37,63] and disruption of chitin synthases has been shown to affect growth of fungi at temperatures of infection.[37] Recently, the assimilation of alkylbenzenes, which occur as environmental pollutants but also in vertebrates as neurotransmitters, has been suggested as a virulence determinant specific to *Chaetothyriales*.[50]

Initial diagnoses in the seadragon cases in the present study were made from gross lesions observed in live and dead animals, cytologic examination of affected tissues, and characteristics of fungi in histologic sections. In instances in which frozen or fresh tissues representing suspected lesions were available, fungal culture of the kidney, skin, liver, and/or spleen consistently yielded velvety, olivaceous, or brown-black molds, which upon further molecular testing were identified as species of *Exophiala*. The genus is morphologically characterized by the presence of annellated zones producing annelloconidia from nearly undifferentiated conidiogenous cells. However, based on rDNA ITS sequence data, it was found that *Veronaea botryosa*, with large conidiophores and 2-celled sympodial conidia, is a member of this clade. Given the fact that the entire clade shows an association with watery environments (ranging from ocean water to drinking water) at cold to mild temperatures, it was concluded that ecology is a prime factor in the phylogeny of these species. Species found in somewhat warmer environments, such as bathrooms and swimming pools, are recurrently encountered as agents of mild human disease, infecting external body parts, such as skin of the extremities, and occasionally nasal sinuses (de Hoog, personal communication, 2008).

Such bacteria as *Vibrio* spp., *Pseudomonas* spp., and *Mycobacterium* spp. are commonly found in sea water and likely represent opportunistic invaders or potential members of normal skin flora.[7,43] Although a variety of Gram-negative bacteria were isolated from skin lesions, no species of bacteria was consistently isolated. Cutaneous lesions in the seadragons were often advanced when examined histologically so that a determination as to whether the inciting lesion was bacterial, fungal, or traumatic was not possible. In contrast, visceral lesions and vascular invasion were fungal in nature, and bacteria were not identified.

To the authors' knowledge, this is the first report describing disseminated phaeohyphomycosis with isolation of *Exophiala* spp. in seadragons. Seadragon habitats, such as algal covered reefs and seagrass meadows, are being adversely affected by human activities, and loss in quality and quantity of habitat has been documented (http://www.dragonsearch.asn.au; www.iucn.org).[14,15,58] Seadragons are difficult to culture and are susceptible to stress of confinement, poor diet, and trauma. Disseminated *Exophiala* spp. infection, as described in these cases, poses challenges to the management and conservation efforts of these fish.

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Sources and manufacturers

a. Finquel/MS-222, Argent Chemical Laboratories Inc., Redmond, WA.
b. Pfizer Inc., New York, NY.
c. Janssen Pharmaceutica, Beerse, Belgium.
d. Fisher Scientific Co., Pittsburgh, PA.
e. National Fish Pharmaceuticals, Tucson, AZ.
f. Virkon®, Antec International Ltd., Sudbury, Suffolk, UK.
g. GlaxoSmithKline, Brentford, Middlesex, UK.
h. Sigma-Aldrich, St. Louis, MO.
i. Northeast Laboratory Services, Waterville, ME.
j. Hardy Diagnostics, Santa Maria, CA.
k. Applied Biosystems, Foster City, CA.
l. Invitrogen Corp., Carlsbad, CA.
m. PTC-100, MJ Research, Inc., Waltham, MA.
n. Applied Maths NV, Sint-Martens-Latem, Belgium.

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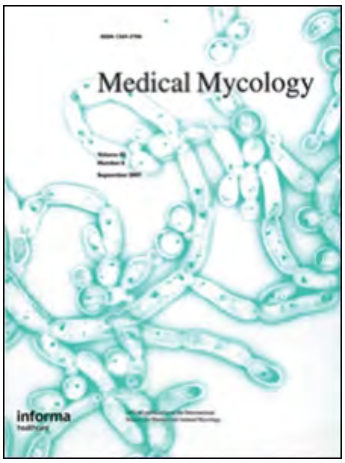

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David C. Grant [a]; Deanna A. Sutton [b]; Christina A. Sandberg [a]; Ronald D. Tyler Jr [c]; Elizabeth H. Thompson [b]; Anna M. Romanelli [d]; Brian L. Wickes [d]
[a] Department of Small Animal Clinical Sciences, [b] Department of Pathology, Fungus Testing Laboratory, [c] Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, Virginia [d] Department of Microbiology, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA

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Disseminated *Geosmithia argillacea* infection in a German Shepherd dog

DAVID C. GRANT*, DEANNA A. SUTTON†, CHRISTINA A. SANDBERG*, RONALD D. TYLER JR.‡, ELIZABETH H. THOMPSON†, ANNA M. ROMANELLI§ & BRIAN L. WICKES§

**Department of Small Animal Clinical Sciences, ‡Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, Virginia, †Fungus Testing Laboratory, Department of Pathology, and §Department of Microbiology, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA*

We report a systemic mycosis in a German Shepherd dog caused by *Geosmithia argillacea*. Although this etiologic agent microscopically resembles a *Penicillium* species, and is histopathologically compatible with members of the genus *Aspergillus*, morphologic features and molecular characterization clearly separate it from these genera. This appears to be the first report of disseminated disease by this species in humans or animals. *In vitro* antifungal susceptibility testing suggests resistance to amphotericin B and voriconazole and susceptibility to caspofungin, itraconazole, and posaconazole.

Keywords *Geosmithia argillacea*, German Shepherd dog

Introduction

Disseminated opportunistic mycoses are infrequently reported in dogs. The most common etiologic agents are species of *Aspergillus,* particularly *A. terreus* and *A. deflectus* [1–12]. There are rare reports of disseminated disease caused by other hyaline genera such as *Penicillium* [13], *Paecilomyces* [14], *Sagenomella* [15], and agents of adiaspiromycosis [16], as well as isolated reports of systemic phaeohyphomycosis [17]. The majority of these opportunistic infections have occurred in German Shepherd dogs leading to suspicion of a breed-related immunodeficiency, although studies by Day *et al*. failed to identify the specific defect [3]. In fact, German Shepherd male dogs have an odds ratio of 49 for disseminated aspergillosis relative to a background hospital population, and female dogs have an odds ratio of 2.9 [12]. Some of the manifestations of these disseminated mycoses in dogs have included discospondylitis, osteomyelitis, spinal hyperpathia, paralysis, pyrexia, weight loss, anorexia, uveitis, endophthalmitis, lameness, head tilt, nystagmus, renal failure, and urinary incontinence. Response to therapy with amphotericin B or triazole antifungals has been marginal. We report here the first case of disseminated infection with *Geosmithia argillacea*.

Case report

A 4-year-old female, spayed German Shepherd dog presented to the Virginia-Maryland Regional College of Veterinary Medicine in February 2008, for evaluation of acute onset glaucoma of the right eye. Moderate aqueous flare and cells, iris bombe, and preiridal membrane were noted on slit lamp biomicroscopy. Vitreal debris and exudative retinal detachment were noted on ocular ultrasonography. The intraocular pressure was 27 mmHg by rebound tonometry. Panuveitis and secondary glaucoma of the right eye were diagnosed. There were no abnormalities detected in the left eye. Topical prednisolone acetate, timolol maleate, and dorzolamide and oral carprofen were prescribed. Due to the combined presence of lethargy, spinal hyperpathia, and panuveitis, an underlying systemic disease was suspected as the cause of the ocular abnormalities and thus the dog was further evaluated. Negative antibody titer results were obtained for *Leptospira* species and *Brucella canis* (Virginia Department of Agriculture and Consumer Services, Wythe-

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Correspondence: David C. Grant, Duck Pond Dr., Department of Small Animal Clinical Sciences, Virginia Tech, Blacksburg, VA 24061-0442, USA. Tel: +1 540 231 5699; Fax: +1 540 231 7367; E-mail: dgrant@vt.edu

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ville, VA) and *Aspergillus fumigatus* (University of Tennessee Veterinary Medical Laboratory, Knoxville, TN). Urine was negative for *Blastomyces dermatitidis* antigen (MiraVista Diagnostics, Indianapolis, IN). Results of a complete blood cell count and biochemical profile were unremarkable. Hematuria and pyuria were noted, but a urine aerobic bacterial culture was negative. Thoracic radiography revealed normal cardiac and pulmonary structures. Radiography of the spine revealed osseous proliferation with concurrent lysis of the vertebral endplates of thoracic vertebrae four, five and six consistent with discospondylitis. Similar changes were noted in multiple sternebrae. Fine-needle aspirates of these sternebrae were evaluated cytologically and yielded peripheral blood only. Ultrasonography of the abdomen revealed bilateral renal pelvic dilation with all other organs appearing normal. In March 2008 the dog was evaluated for response to ocular medications and to further pursue the cause of discospondylitis. A previously undetected systolic ejection murmur was ausculted over the left heart base. Echocardiography identified a small patent ductus arteriosus, but no valvular lesions suggestive of endocarditis or cause for the ejection murmur were found. The dog was blind in the right eye with end-stage glaucoma with buphthalmos. The intraocular pressure was 50 mmHg by rebound tonometry. Rubeosis iridis and posterior and peripheral anterior synechiae of the iris were noted in the right eye. The left eye had fibrin strands in the anterior chamber and multifocal chorioretinitis in the tapetal fundus. Enucleation of the right globe for histopathologic diagnosis was performed. Fluoroscopic-guided core biopsies of multiple sternebrae were obtained. Aerobic bacterial cultures of a vitreal aspirate, sternebral biopsy, and urine were negative. Carprofen and tramadol were given post-operatively for pain control. Histopathologic evaluation of the eye identified lymphoplasmacytic panuveitis, intra-retinal hemorrhage, lens capsule rupture with pyogranulomatous inflammation, and retinal detachment with exudative vitreitis. Histopathologic evaluation of the sternebrae by hematoxylin and eosin staining revealed mild lamellar bone resorption with fibrous replacement. Fungal Gomori methenamine silver (GMS) stains revealed septate, dichotomously branching hyphae measuring 3–5 μm in diameter within the lens, retina, and sternebrae (Fig. 1 & 2). *Aspergillus terreus* was suspected based on histopathology compatible with aspergillosis and the reported prevalence of this organism in German Shepard dogs. A urine sample was obtained by cystocentesis and inoculated onto Sabouraud dextrose agar (SDA) (Remel, Lenexa, KS). The

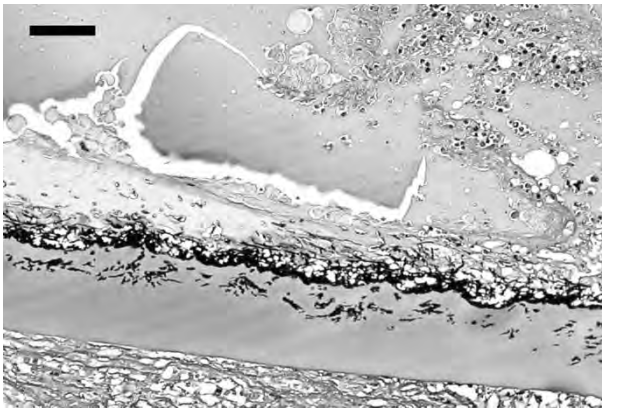

Fig. 1 GMS stain, eye, (bar equals 50 microns). Multiple septate hyphae invading the anterior lens capsule and lens cortical material.

microscopic morphology of the isolate grown on this medium after 14 days incubation at 30° C resembled a *Penicillium* species, although the isolate was subsequently identified as *Geosmithia argillacea* at the University of Texas Health Science Center at San Antonio (UTHSC). No antifungal treatment was administered. Over the next month the dog became increasingly agitated and developed a head tilt and nystagmus. Examination of the left eye revealed more severe posterior segment disease, with vitreal debris, chorioretinal scarring and focal retinal detachment. Humane euthanasia was elected and a necropsy was performed.

At necropsy the pleural surfaces were red and granular, and multiple 0.5–1mm nodules were dispersed throughout the lungs. The liver was diffusely congested and slightly enlarged. The kidneys were irregular and red and contained multifocal, small,

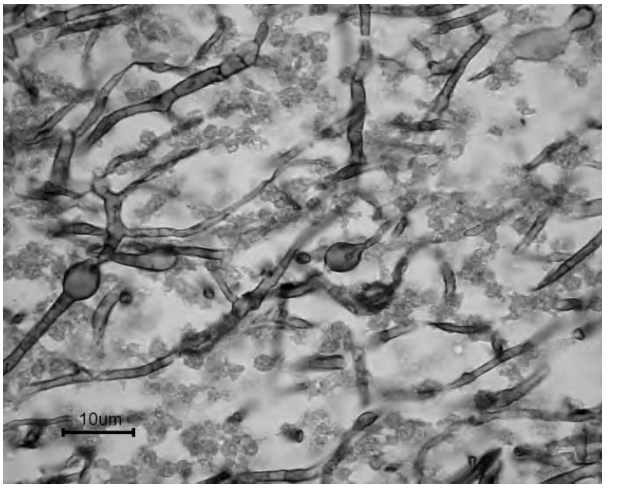

Fig. 2 GMS stain, sternebra, (bar equals 10 microns). Multiple septate hyphae with bulbous endings are dispersed throughout.

white-tan, granular nodules most prominent along the pelvises (Fig. 3). The spleen was diffusely enlarged and mottled red-white. The third, fourth, and fifth sternebrae were enlarged with a firm proliferation between the articular surfaces. The bodies of the sternebrae were osteolytic and filled with a brown-tan granular caseous material. The ventral aspects of the fifth, sixth, and seventh thoracic vertebrae were thickened with firm nodules along the articular surfaces. There was marked osteolysis of the central vertebral bodies and they were filled with a white caseous material. The right cerebrum of the brain was moderately firm but the remainder of the central nervous system was unremarkable. Microscopically the lungs, pancreas, liver, kidney, and cerebrum had multifocal regions of granulomatous inflammation often associated with blood vessels. Some granulomas from each of these organs were centrally necrotic and contained septate, dichotomously-branching fungal hyphae with bulbous ends (Fig. 4). There was extensive fibrosis around regions of inflammation within the pancreas and kidneys. The affected sternebrae and thoracic vertebral bodies also had extensive osteolysis, fibrosis, necrosis and multifocal regions of granulomatous inflammation that crossed articular surfaces. Similar hyphae were seen within necrotic regions of bone. Gomori methenamine silver stains documented hyphae in all affected tissues. Tissue samples from the left cerebrum and cerebellum, affected sternebrae and vertebrae, kidney, and bladder were inoculated onto SDA. With the exception of brain tissue, all other samples grew a fungus morphologically identical, both macroscopically and microscopically, to the urine isolate previously identified as *Geosmithia argillacea*. Molecular confirmation of the same organism from both the urine and the vertebra confirmed *Geosmithia argillacea* as the etiologic agent of disseminated disease.

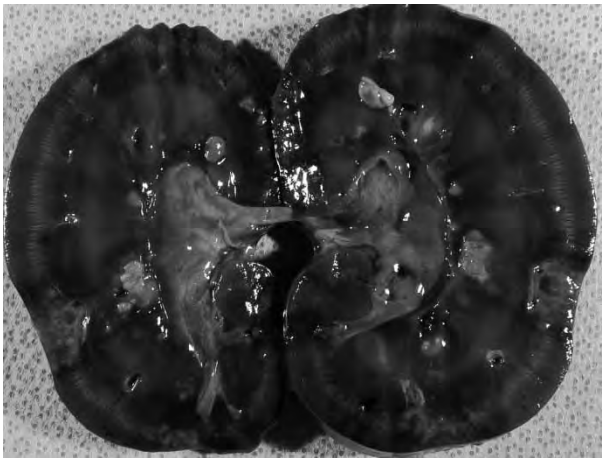

Fig. 3 The kidney is irregular and red with multifocal, large, white-tan, granular nodules most prominent along the renal pelvis. There is a wedge shaped pale area extending from the cortex to the medulla consistent with an infarct.

Identification of the etiologic agent

Both the urine isolate and the necropsy thoracic vertebra isolate were forwarded to the Fungus Testing Laboratory for molecular and morphologic characterization and were accessioned into their stock collection as UTHSC R-4148 and R-4234, respectively. Isolates were grown for 20 h at 30°C on potato dextrose agar (Difco, Detroit, MI). A small amount of hyphae was removed and suspended in 50 µl of Prepman Ultra reagent (Applied Biosystems, Foster City, CA) in a 0.5 ml microfuge tube. The suspension was heated for 15 min at 100°C and then pelleted for 5 min at 14, 000 *g* in a microfuge according to the manufacturer's instructions. PCR reactions were performed directly on 5 µl of the Prepman supernatant in a 50 µl reaction using TripleMaster *Taq* DNA polymerase (Fisher Scientific, Pittsburgh, PA) according to the manufacturer's instructions. ITS amplicons were obtained using primers (ITS1 and ITS4) and PCR conditions as previously described [18]. D1/D2 PCR amplicons were obtained using primers (NL-1 and NL-4) and PCR conditions as described [19,20]. Amplifications were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA) and amplicons of the expected size were visualized by running a 15 µl aliquot of each PCR reaction on a 0.7% agarose gel followed by staining with ethidium bromide and viewed by ultraviolet transillumination. The remaining PCR template was prepared for sequencing by cleaning with a QIAquick PCR purification column (Qiagen, Valencia, CA). Purified templates were sequenced at the UTHSCSA Advanced Nucleic Acids Core facility using the same primers for ITS and D1/D2 amplification. Sequences were then used to perform individual BLASTn (Basic Local Alignment Search Tool) searches using the NCBI (National Center for Biotechnology Information) BLAST database. Genbank accession numbers were assigned as follows: R-4148 ITS, D1/D2 (ACCESSION# EU862335, ACCESSION#EU862336), R-4234 ITS, D1/D2 (ACCESSION# EU862337, ACCESSION# EU862338). A BLASTn search of the R-4148 and R-4234 ITS and D1/D2 sequences returned identical results. The three highest% identities for the ITS region were: (1) *Geosmithia argillacea* 525/541 (97%) accession #AF033389, (2) *Talaromyces eburneus* (the teleomorph of *Geosmithia argillacea*) 461/477 (96%) accession #AB176614, and (3) *Monascus fumeus*

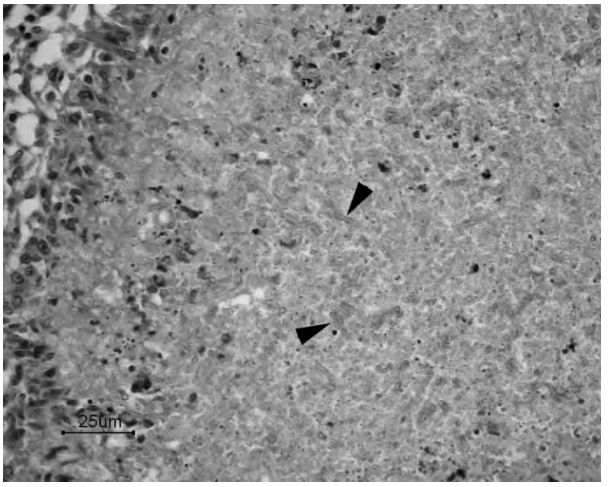

Fig. 4 H&E, kidney (bar equals 25 microns). The centers of granulomas are necrotic and contain poorly staining septate, dichotomous branching fungal hyphae (arrowheads) with bulbous endings.

508/584 (86%) accession # DQ978996. Analysis of the Genbank alignments revealed that the mismatches were in both the ITS1 and ITS2 regions. No mismatches occurred in the 5.8s rDNA region. The three highest% identities for the D1/D2 sequence were: (1) *Geosmithia argillacea* 614/614 (100%) accession # AB047236, (2) *Geosmithia argillacea* 614/614 (100%) accession # AB047235, and (3) *Geosmithia argillacea* 613/614 (99%) accession # AB047238.

The macroscopic morphology of *G. argillaceae* on malt extract agar (MEA) (Remel, Lenexa, KS, dehydrated and prepared in-house) is depicted in Fig. 5A (16 days at 23°C) and 5B (8 days at 35°C). Growth was slow and restricted at the lower temperature, attaining 21-23 mm in diameter after 16 days as compared to 34–36 mm in 8 days at the higher temperature. Colonies at 23°C were cream to buff-colored with ill-defined margins while those at 35°C were similarly colored with entire margins. Reverse and obverse colony colors were the same. Temperature studies conducted on potato flakes agar (PFA) tubed media, prepared in-house, demonstrated good growth at 37, 40, and 45°C but no growth at 50°C. Maximum growth temperatures are presumed to be near 50°C based upon our studies and those of earlier investigators [21,22]. Microscopic features observed from a PFA slide culture preparation included rough, hyaline, septate, stipes, often branched, ranging from 70–200 μm in length, penicilli that were monoverticillate to biverticillate (asymmetric) to terverticillate, cylindrical, appressed, slightly roughened phialides measuring10–12 × 2–3μm and tapering at the apex, and smooth hyaline conidia borne in long, columnar chains. Conidia, measuring 2.5–5 × 1.5–2.5 μm, were initially cylindrical to cuniform (wedge-shaped) and became ellipsoidal to ovoid at maturity (Fig. 6). Based on the sequence identities and the morphologic features, both isolates were identified as *Geosmithia argillacea* and have been deposited into the University of Alberta Mold Herbarium under the accession numbers UAMH 10932 (R-4148, urine) and UAMH 10933 (R-4234, vertebra).

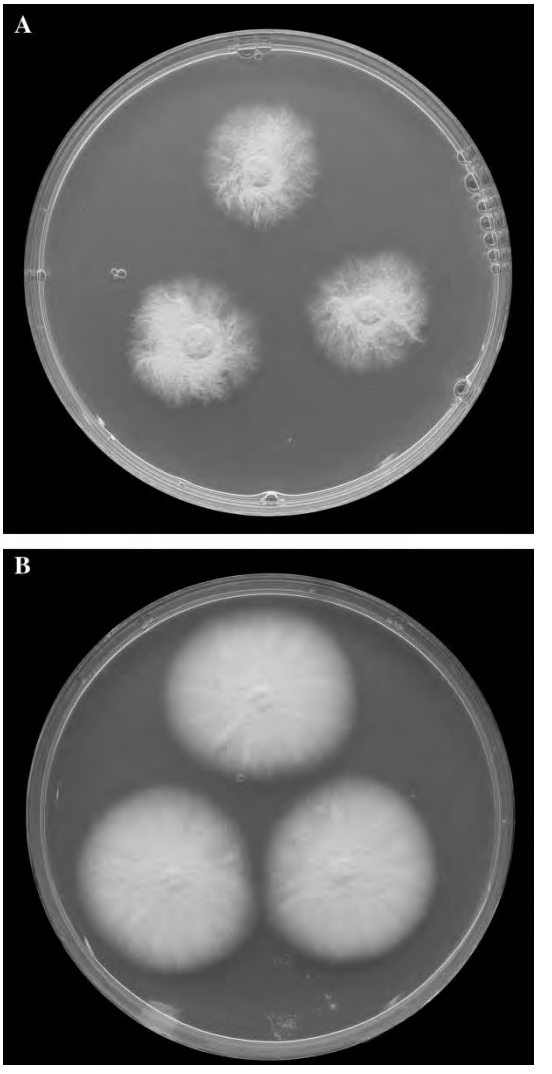

Fig. 5 Macroscopic morphology of *Geosmithia argillacea* on malt extract agar. (A) 16 days at 23°C. (B) 8 days at 35°C.

In vitro antifungal susceptibility testing

Antifungal susceptibility testing of *G. argillacea* was performed on the isolate from the vertebra. It was accomplished in a macrobroth dilution format in essential agreement with the previously published Clinical and Laboratory Standards Institute document

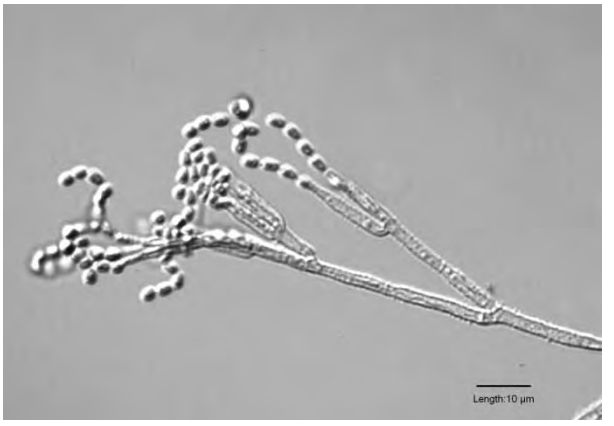

Fig. 6 Microscopic morphology of *Geosmithia argillacea* demonstrating branching stipes, monoverticillate and asymmetric biverticillate penicilli, cylindrical and appressed phialides, and smooth, hyaline, cuniform to ellipsoidal conidia borne in long, columnar chains. Roughened stipes, metulae, and phialides are a distinctive microscopic feature of this species (bar equals 10 microns).

M38-A [23]. Amphotericin B (AMB, Bristol-Meyers, Squibb, New York, NY) and caspofungin (CAS, Merck, Rahway, NJ) were tested in Antibiotic Medium 3 (Difco, Sparks, MD) while, voriconazole (VRC, Pfizer, Inc., New York, NY), itraconazole (ITC, Janssen Pharmaceutica, Piscataway, NJ) and posaconazole (PSC, Schering Plough, Galloping Hill, NJ) were tested in RPMI-1640 (Hardy Diagnostics, Santa Maria, CA). Concentrations tested for all drugs ranged from 0.03 to 16 µg/ml. Tubes were incubated at 35°C and were read against a positive growth control tube at either 24 and 48 h (AMB and CAS) or 48 and 72 h (ITC, VRC, PSC), depending upon the growth rate of the organism in the test medium. Endpoints for AMB were the lowest concentration that inhibited visual growth, while those for the triazoles (ITC, VRC, PSC) were 80% inhibition compared to the growth control. Caspofungin endpoints were read as minimum effective concentrations (MECs) [24,25]. Results for AMB and CAS were 1 and 2, and 0.125 and 0.25 µg/ml, respectively. Results for the triazoles were 0.25 and 0.25, >16, and 0.06 and 0.06 µg/ml for ITC, VRC, and PSC, respectively. No defined breakpoints are currently available for these antifungal agents against this organism.

Discussion

The genus *Geosmithia* currently contains numerous species formerly classified as *Penicillium*. *Geosmithia argillacea* (Stolk, H.C. Evans & T. Nilsson) [26], was originally described as a new thermotolerant *Penicillium* species by Stolk *et al*. who isolated the type strain from a high-temperature mine waste tip in 1969 [21]. In 1979 Pitt [26] erected the genus *Geosmithia* to distinguish isolates previously known as *Penicillium* spp. but which formed conidia borne as cylinders from cylindrical, rough-walled phialides lacking narrow necks, as in *Penicillium* and *Paecilomyces*, and that produced conidia that were not typically some shade of green. In 1994, Yaguchi *et al*. [27] described a new species of *Talaromyces, T. eburneus,* from the soil in Taiwan. In a subsequent investigation [28] of an outbreak of fungal contamination of pasteurized pineapple juice in the beverage industry, he recovered a strain of *Talaromyces eburneus* having a *Geosmithia* anamorph (asexual form). As this species had not been previously regarded as thermophilic, sequence analysis was performed to compare this species with the type strain of *T. eburneus*, and 3 strains of *Geosmithia argillacea*. The D1/D2 regions of 28S rDNA for all strains were identical, thereby confirming *T. eburneus* as the teleomorph (sexual form) of *Geosmithia argillacea* [22,28]. The etiologic agent in the dog in the current report was initially thought to be a *Penicillium* species based on its microscopic morphology, however a more detailed examination of the morphologic features combined with molecular characterization confirmed the identification as *G. argillacea* and emphasizes the utility of ITS and D1/D2 sequencing. Previous reports of disseminated infection with *Penicillium* species may have suffered from similar misidentification.

To our knowledge this is the first report of a *Geosmithia* species causing disseminated disease in either humans or animals. *Geosmithia argillacea* was isolated from a pleural cavity drain from a human, though the method of determining fungal identity was not described [29]. More recently, *G. argillacea* has been considered a potential pathogen in cystic fibrosis lung disease [30]. The breed and gender of the dog and the physical manifestations of infection with *Geosmithia* in this report were typical of those associated with disseminated aspergillosis [12]. We suspect this dog may have had a predisposing immunodeficiency, though tests of immune function were not performed due to financial constraints. Antifungal therapy was not administered as prognosis for other seemingly similar disseminated mycoses such as aspergillosis and penicilliosis is so poor. *In vitro* antifungal susceptibility testing performed post-mortem suggested susceptibility to itraconazole, posaconazole, and caspofungin raising the possibility that treatment may have had a beneficial effect. The ability of these drugs to penetrate all infected tissues, however, is questionable. Amphotericin B may also have been efficacious had a liposomal

preparation been used, while voriconazole clearly lacked activity, *in vitro*.

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Strain-Dependent Variation in 18S Ribosomal DNA Copy Numbers in *Aspergillus fumigatus*▽

M. L. Herrera,[1] A. C. Vallor,[2] J. A. Gelfond,[3] T. F. Patterson,[2] and B. L. Wickes[1,4]*

Department of Microbiology and Immunology,[1] Department of Medicine,[2] and Department of Epidemiology and Biostatistics,[3] the University of Texas Health Science Center at San Antonio, and the San Antonio Cancer Institute,[4] San Antonio, Texas

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Enumerating *Aspergillus fumigatus* CFU can be challenging since CFU determination by plate count can be difficult. CFU determination by quantitative real-time PCR (qPCR), however, is becoming increasingly common and usually relies on detecting one of the subunits of the multicopy rRNA genes. This study was undertaken to determine if ribosomal DNA (rDNA) copy number was constant or variable among different *A. fumigatus* isolates. *FKS1* was used as a single-copy control gene and was validated against single-copy (*pyrG* and *ARG4*) and multicopy (*arsC*) controls. The copy numbers of the 18S rDNA subunit were then determined for a variety of isolates and were found to vary with the strain, from 38 to 91 copies per genome. Investigation of the stability of the 18S rDNA copy number after exposure to a number of different environmental and growth conditions revealed that the copy number was stable, varying less than one copy across all conditions, including in isolates recovered from an animal model. These results suggest that while the ribosomal genes are excellent targets for enumeration by qPCR, the copy number should be determined prior to using them as targets for quantitative analysis.

Aspergillosis is caused by pathogenic fungi in the genus *Aspergillus* and includes allergic, superficial, saprophytic, and invasive disease (12). The frequency of invasive aspergillosis (IA) continues to increase due to a growing population of immunosuppressed individuals. In fact, *Aspergillus fumigatus*, the most frequent *Aspergillus* species in IA cases (19), is now the most common airborne human fungal pathogen (25). The mortality rate for IA can be unacceptably high for some patient populations, once infected, ranging from 70 to 90%, depending on the patient type (7, 13, 31). However, in spite of the severity of disease, the ubiquitous nature of *Aspergillus* in the environment makes exposure difficult to avoid; consequently, susceptible patients will almost always be at risk for infection.

The life-threatening nature of IA makes accurate diagnosis and early detection crucial. Quantitative real-time PCR (qPCR) is emerging as a sensitive and cost-efficient technique for detecting *Aspergillus* spp. from a diverse variety of sources, including clinical specimens. Investigators studying IA with animal models routinely use qPCR to measure fungal load (17, 27), including response to drug treatment (6, 42). Bioaerosol quantitation of *Aspergillus* spp., particularly in the hospital environment, is also amenable to qPCR (32). Finally, even though qPCR is not the first choice for clinical diagnosis of IA, it has proven useful for quantitating *Aspergillus* spp. from a variety of patient specimens (2, 26, 38) and has proven extremely useful as a secondary assay for comparative purposes during assay development (8, 23).

One of the drawbacks of PCR-based detection methods is a lack of standardization (5), and one of the first areas to standardize is selection of an appropriate target for amplification. The quantitative nature of qPCR allows an estimation of the number of CFU by equating the copy number of the target sequence with the genome number through a simple ratio, provided the ratio remains invariant. With fungi, the ribosomal genes have proven to be useful PCR targets because of their sequence conservation, which has allowed the use of universal primers that enable the amplification of targets from unknown species. A second advantage of using the ribosomal DNA (rDNA) genes as an amplification target is the copy number, which can be 10 to 100 times that of single-copy genes (29, 30). However, in *A. fumigatus*, it is unclear whether all strains have the same number of rDNA subunits. With other fungi, the rDNA copy number is known to vary (4, 15, 16, 20, 29), although these observations have been made with fungi that are not frequently recovered as human pathogens. Given what is known for other organisms about the variability of the rDNA copy number and the importance of *A. fumigatus* as a human pathogen, this study was performed in order to determine if rDNA copy number is constant or strain specific in *A. fumigatus*.

MATERIALS AND METHODS

Strains and media. Strains used in this study are shown in Table 1 and were confirmed to be *A. fumigatus* by colony morphology and DNA sequencing of the internal transcribed spacer (ITS) and D1/D2 regions. Each strain was grown on Sabouraud's dextrose (Difco Laboratories, Detroit, MI) or potato dextrose broth or potato dextrose agar (PDA) (Fisher Scientific, Pittsburgh, PA) for all assays, unless otherwise indicated. Agar media were prepared from broth by solidification with 2% agar. RPMI 1640 without L-glutamine (Mediatech, Inc., Herndon, VA) was prepared by filter sterilizing and added to an autoclaved solution of 2% dextrose and 2% agar (BD Diagnostic Systems, Franklin Lakes, NJ).

DNA isolation. Individual strains were inoculated into 200 ml of Sabouraud's dextrose broth in a 500-ml flask from a 7-day-old suspension of ~5×10^8 conidia harvested from a PDA plate. The hyphae were recovered after 24 h by filtering through an 18.5-cm, 0.45-mm-pore-size Whatman disk (Whatman, Florham Park, NJ) and washed with sterile saline. DNA isolation consisted of methods reported elsewhere (22, 41), with slight modifications. After the saline wash,

* Corresponding author. Mailing address: Department of Microbiology and Immunology, the University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., Mail Code 7758, San Antonio, TX 78229-3900. Phone: (210) 567-3938. Fax: (210) 567-6612. E-mail: wickes@uthscsa.edu.

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TABLE 1. Strains used in this study[a]

Strain[b]	Contributor, strain alias, and/or reference
AF293	R. Aramayo
WSA-172	M. Rinaldi, no. 98-407
WSA-270	ATCC 64746
WSA-271	ATCC 14110
WSA-419	K. J. Kwon-Chung, no. B-5233, 40
WSA-445	T. Patterson, no. MTFP0009
WSA-446	M. Rinaldi, no. 99-1900
WSA-621	B. Lutz, no. 1

[a] All strains were obtained from clinical sources.
[b] WSA isolates are from the Wickes laboratory culture collection.

approximately 200 mg of wet hyphae were briefly dried by blotting between Whatman paper (Whatman) and then placed into a sterile mortar and frozen for 10 min at −70°C. Fungal cell walls were mechanically broken by grinding with a pestle for 1 to 2 min after the addition of sterile sand and 2 ml of Masterpure yeast DNA purification kit lysis buffer (Masterpure yeast DNA purification kit; Epicentre Technologies, Madison, WI). The slurry was transferred to 2- by 1.5-ml Microfuge tubes and spun at low speed (500 × *g*) for 15 s to pellet the sand. Four hundred microliters of the supernatant were transferred to a 2.0-ml screw-cap Microfuge tube and incubated at 65°C for 2 h, after the addition of 6 μl of proteinase K (50 μg/ml) from the DNA purification kit. Samples were processed from this point as described previously (22). After the final wash, the dried pellets were resuspended in 200 μl ultra pure water (Invitrogen, Carlsbad, CA). DNA was assessed for quality and quantified by gel electrophoresis and a 260-nm/280-nm absorbance ratio.

Due to the possibility of contamination of *Aspergillus* DNA with polysaccharides in crude DNA preps, DNA was further purified prior to performance of qPCR assays. DNA was run in a 1.0% low-melting-point agarose (InCert; FMC BioProducts, Rockland, ME) gel to separate it from contaminating materials. Gel fragments containing DNA were recovered, placed into 1.5-ml Microfuge tubes, and then treated with Gelase (Epicentre) according to the manufacturer's instructions. Purified DNA was assessed and quantitated by spectrophotometer and agarose electrophoresis as described above. Yields were 100 μg to 500 μg.

Growth conditions to evaluate stress effect on rDNA copy number. In order to measure the effect of colony age on rDNA copy number, DNA was prepared from *A. fumigatus* strain AF293 grown for 3 days, 5 days, 10 days, and 25 days on PDA plates at 30°C. AF293 was also tested for the effect of temperature on copy number by preparing DNA from cultures grown at 30°C and 45°C for 5 days on PDA plates. DNA was isolated and processed from each condition, as previously described (22).

The effect of antifungal exposure on copy number was measured by harvesting AF293 grown in the presence of itraconazole (Oakdell Pharmacy, San Antonio, TX) using a modification of the standard MIC assay. Conidia were harvested from a 5-day-old PDA plate grown at 30°C overnight and used to prepare inoculums containing 4.5×10^6 CFU/ml. Each inoculum (10 ml) was then grown overnight at 30°C in the presence of different itraconazole concentrations (0 μg/ml, 0.03 μg/ml, 0.06 μg/ml, 0.125 μg/ml, 0.25 μg/ml, 0.5 μg/ml, 1.0 μg/ml, and 2.0 μg/ml) under modified MIC conditions described by the National Committee for Clinical Laboratory Standards (33). DNA was then recovered as described above.

In order to determine what effect morphology had on copy number, AF293 DNA was isolated from pure conidia and hyphae. Conidial cultures were prepared from PDA plates grown for 11 days at 30°C and harvested by washing with 10 ml of sterile PBS-0.1% Tween 20. The suspension was pelleted by centrifugation at 4,800 × *g* for 10 min. The supernatant was discarded, and the conidial pellet was transferred to a 1.7-ml microcentrifuge tube and washed once with 500 μl of sterile water and once with 500 μl of 0.1 M $MgCl_2$. Hyphae were prepared as described previously (22). Conidial and hyphal DNA were recovered as described above.

The effect of growth in vivo during animal model infection on copy number was determined by passing AF293 through animals as follows. Nonimmunosuppressed mice and guinea pigs were infected as described by Sheppard et al. (35). Lungs and kidneys were harvested 5 days postinfection. DNA was extracted from tissue according to the Standard Operating Procedures for Invasive Aspergillosis Animal Models (http://www.sacmm.org/sop.html) and recovered in 100 μl of QIAamp DNA minikit elution buffer (Qiagen, Valencia, CA). After quantitation, DNA was stored at −20°C until analyzed.

PCR and qPCR primer and probe design. The PCR primer and probe sequences used to quantitate and amplify *A. fumigatus* target genes are shown in Table 2. Primers for qPCR were designed using Primer Express software version 2.0, which is application-based design software provided by ABI (Applied Bio-

TABLE 2. PCR primer and probe sequences

Primer or probe[b]	Sequence	Reference or source
18S rDNA.F	5′-GGCCCTTAAATAGCCCGGT-3′	10
18S rDNA.R	5′-TGAGCCGATAGTCCCCCTAA-3′	10
18S rDNA probe[a]	6-FAM-AGCCAGCGGCCCGCAAATG-MGBNFQ	10
AFKS.F	5′-GCCTGGTAGTGAAGCTGAGCGT-3′	6
AFKS.R	5′-CGGTGAATGTAGGCATGTTGTCC-3′	6
AFKS probe	6-FAM-TCACTCTCTACCCCCATGCCCGAGCC-MGBNFQ	6
AFKS probe	6-VIC-TCACTCTCTACCCCCATGCCCGAGCC-MGBNFQ	6
ARG4.F	5′-CAGCCCCGGGAAACTCA-3′	This study
ARG4.R	5′-TCCGCTCCCTTGACAGCTT-3′	This study
ARG4 probe	6-FAM-CCAGACCAATGTTCCTGAG-MGBNFQ	This study
pyrG.F	5′-TGGCCCAGACCGCATCT-3′	This study
pyrG.R	5′-CAACAGTCCTCTCTCAGGACCAT-3′	This study
pyrG probe	6-VIC-CGCAAGACTTCCC-MGBNFQ	This study
arsC.F	5′-GCCGCTGGGTTCCTTACTC-3′	This study
arsC.R	5′-CAGCGGAGCGAACCTCAATA-3′	This study
arsC probe	6-FAM-CCTCGCAGGTGATG-MGBNFQ	This study
Chr1arsC.F	5′-GACCTCGACACCCTAAGAAGC-3′	This study
Chr1arsC.R	5′-TCAAATGATGAGAGGCCAGA-3′	This study
Chr5arsC.F	5′-TCCTCCATCTTCATTCCCTTA-3′	This study
Chr5arsC.R	5′-GAGCTGGAACCTCAGCGTAG-3′	This study

[a] MGB probe dyes are incorporated into the primer sequences, i.e., 6-FAM-AGCCAGCGGCCCGCAAATG-MGBNFQ is an MGB probe labeled with FAM.
[b] AFKS primers and probes were used for detection of the *FKS1* gene. *ARG4*, *pyrG*, and *arsC* primers and probes were used for detection of the *ARG4*, *pyrG*, and *arsC* genes. Primers designated Chr1arsC or Chr5arsC are for routine PCR amplification of the two *arsC* alleles from chromosome 1 or 5.

systems, Inc., Foster City, CA), or were designed based on previously published reports. The primers and probe for the *A. fumigatus FKS1* gene were designed according to Costa et al. (10). The primers and probe for the 18s rDNA sequence were also based on a previous study (6). The *FKS1* gene was chosen because it is a known single-copy gene in *A. fumigatus* involved in β(1–3) glucan synthesis (3) and was used as an internal control. The *pyrG* gene, which encodes orotidine-5′-monophosphate decarboxylase, was also included as a second single-copy reference gene (11, 44) and used to confirm *FKS1* copy number determination. *ARG4*, which encodes carbamoyl-phosphate synthase, was the third single-copy reference gene used in this study and was identified from the genome sequence.

In order to test our ability to discriminate multiple copy genes, a duplicated gene was selected for analysis. The *arsC* (arsenate reductase) gene is a duplicated gene found in some but not all strains of *A. fumigatus* and is present in the AF293 genome sequence as two copies (34). Since *arsC* is not present in all *A. fumigatus* strains, we reconfirmed that it was present in AF293 in two copies by use of a method independent of qPCR. Based on the sequences of *arsC* from the two chromosomal locations, allele-specific primers were designed that spanned an NcoI site within the coding sequence of each *arsC* allele (Table 2). The chromosome 1 *arsC* primers consisted of Ch1arsC.F and Ch1arsC.R. The chromosome 5 *arsC* primers consisted of Chr5arsC.F and Chr5arsC.R. Each allele was amplified using the following conditions: 94°C for 2 min; 32 cycles at 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s; and a final extension of 72°C for 2 min. The amplicons were then digested with 5 U of NcoI (New England Biolabs, Beverly, MA) at 37°C for 3 h and then separated on a 3% NuSieve GTG agarose gel (Cambrex Bio Science, Inc., Rockland, ME). Sizes were then compared to the sizes predicted from the genome sequence.

qPCR validation assays and calculations. *FKS1*, *pyrG*, *ARG4* (single-copy genes), *arsC* (two-copy gene), and 18S rDNA (multiple copies) gene copy number determinations were done by qPCR (TaqMan) assay according to the method of Townson et al. (39), with modifications. In order to determine the copy number of a variable gene (18S rDNA), a single-copy reference gene needed to be identified and confirmed to be present in one copy/genome. Since the *FKS1* gene is highly conserved in fungi and has been shown in a number of reports to be present in single copy in *A. fumigatus* (3, 14, 34), we selected this gene to use as the single-copy reference probe in the quantitative reverse transcription-PCRs. Confirmation was performed by comparison to other *A. fumigatus* genes already known to be single copy. The single-copy genes *pyrG* and *ARG4* were confirmed using relative quantification (ratios of one gene to another) to determine the number of copies present per genome. Quantification standards were run in conjunction with each set of samples after primers and probes for the *FKS1*, 18S rDNA, *pyrG*, *ARG4*, and *arsC* genes were optimized for template concentration and primer efficiencies (1).

qPCRs were performed in triplicate using an ABI Prism 7900 sequence detector system (ABI) to detect minor groove binder probe binding. *FKS1* was quantitated using both VIC and 6-carboxyfluorescein (FAM) dyes and used as a reference for comparison to the 18S rDNA FAM probe from each strain. Six serial 1:2 dilutions (20.0, 10.0, 5.0, 2.5, 1.25, and 0.625 ng/μl) of genomic DNA from *A. fumigatus* AF293 were used to generate standard curves of C_T (threshold cycle) value against the log DNA concentration on each PCR plate for the *FKS1* and 18S rDNA genes. Each experiment was performed three separate times from one DNA preparation and run in duplicate. C_T values were determined and then converted into template quantity. After the creation of standard curves, the copy number of each gene was determined by DNA quantification using TaqMan technology. PCR cycle numbers were plotted against the value of 5′ fluorescence signal, and then threshold values were plotted against the copy number of the template DNA that was used to generate standard curves (1).

Absolute quantification using the ABI Prism 7900 requires that the absolute quantities of the standards be determined by some independent means first. In this study, fungal DNA was used to prepare absolute standards. Concentration and DNA quality were measured by determining the A_{260} and by gel electrophoresis and converted to the number of copies by use of the molecular weight of the DNA. The equation $C_T = m$ (log quantity) + b from the equation for a line ($y = mx + b$) was constructed by plotting the standard curve of log quantity versus its corresponding C_T value. If the curve demonstrated an r^2 value of >0.980, the standard curve then was used to determine sensitivity, primer efficiencies, and dynamic range, as well as specificity and reproducibility of every assay (*FKS1*, 18S rDNA, *pyrG*, *ARG4*, and *arsC*). Amplification of serially diluted genomic DNA (standard curves) from *A. fumigatus* AF293 was repeated in triplicate, on different days, in order to test reproducibility, primer efficiencies, and DNA optimal dilutions for the rest of the genes (*pyrG*, *ARG4*, and *arsC*). DNA concentrations ranged from 20.0 to 0.625 ng/μl. Specificity for all the assays was assessed by using DNA extracted from *Candida albicans* SC5314, as well as mouse and guinea pig DNA (9, 28). Comparative copy numbers for confirmation experiments were determined using the relative quantification ($\Delta\Delta^{C_T}$) $2^{-\Delta\Delta C_T}$ method. The 18S rDNA copy numbers were determined by the absolute quantitation method, by which total copies were first calculated using the following equation: total 18S rDNA copies = $10([C_T - b]/m)$. The number of 18S rDNA copies per genome was then determined by the following equation: 18S rDNA copies per genome = (total copies of 18S rDNA)/(total copies of *FKS1*). Copy number was calculated as the ratio of template quantity for 18S rDNA to the template quantity for *FKS1*.

Statistical methods. In each experiment, we altered one factor at a time under controlled conditions. This approach minimized the sources of variability within an experiment and maximized statistical power for detecting effects of a single factor on differential copy numbers. Results after determination of 18S rDNA copy numbers were compared by the Wilcoxon rank sum test for morphology and temperature. The Wilcoxon signed-rank test was used to compare copy numbers from different tissues in the same animal, and the Kruskal-Wallis test was used to compare culture ages and antifungal susceptibilities. Statistical analysis was done at the University of Texas Health Science Center at the San Antonio Department of Epidemiology and Biostatistics. Two-tailed P values less than 0.05 were considered significant.

RESULTS

Copy number confirmation of *FKS1*. A number of confirmatory assays were performed to verify that *FKS1* was present as a single copy in AF293. First, absolute quantitation was performed using *FKS1* probes labeled with two dyes, FAM and VIC. The slope of the VIC line was −3.9341 (from $y = -39341x + 52.288$), while the slope for the FAM line was −3.8971 (from $y = -3.8971x + 51.593$). The r^2 values of the VIC and FAM lines were 0.9946 and 0.9982, respectively, demonstrating that comparable results could be obtained independently of dye type. The copy number of *FKS1* was next determined in a subset of *A. fumigatus* strains (WSA-172, WSA-445, WSA-621, and WSA-419) by absolute quantitation using *FKS1* labeled with FAM and VIC for each strain. *FKS1* copy numbers determined by qPCR ranged from 0.93 to 1.10 copies and were rounded to 1 copy based on the close integer scoring method (18) so that it could be used as the single-copy reference gene when determining the copy numbers of other genes. We next compared the copy number of *FKS1* to that of other known single-copy genes (*ARG4* and *pyrG*) using absolute quantification. The corresponding calculations of copy numbers of the three genes in AF293 by comparison of the C_T values confirmed that each gene was present in single copy. This outcome was also observed with other *A. fumigatus* isolates (Table 3) and confirmed that *FKS1* was suitable as a single-copy control gene.

Detection of a multicopy gene in *A. fumigatus*. In order to accurately quantitate multicopy genes, it was necessary to demonstrate that *FKS1* could be used to quantitate a multicopy gene of known copy number. Furthermore, we were interested in knowing how discriminatory our strategy would be with regard to copy number accuracy. To make this determination, we decided to use a multicopy gene that was present in low copy number and chose *arsC* as a target. Sequence analysis of *arsC* from the AF293 genome suggested that it was present in two copies, one copy on chromosome 1 and one copy on chromosome 5. Careful inspection of the two sequences by DNA alignment revealed that a combination of primer position and restriction digestion would confirm the presence of two copies, after gel electrophoresis, based on the predicted sizes of digestion products of the PCR (Fig. 1A). The data shown in Fig. 1B confirm that the predicted digestion patterns

TABLE 3. Confirmation of copy number of predicted single-copy genes[a]

Strain	*FKS1* avg C_T	*FKS1* copy no.	*pyrG* avg C_T	*pyrG* copy no.	*ARG4* avg C_T	*ARG4* copy no.
AF293	19.16 ± .012	1	19.33 ± .005	1.13	19.06 ± 0.008	1.07
WSA-172	21.39 ± .012	1	21.34 ± .001	0.97	21.23 ± .007	1.11
WSA-445	22.26 ± .049	1	22.55 ± .003	1.22	22.31 ± .004	0.97
WSA-621	24.34 ± .014	1	23.52 ± .004	1.29	24.54 ± 0.007	1.10
WSA-419	21.40 ± .050	1	21.82 ± .003	1.34	21.40 ± .005	1.00

[a] C_T values are means ± standard deviations (three samples, run in duplicate). The copy number of the test gene (*pyrG* or *ARG4*) was equal to $2^{-\Delta\Delta CT}$.

of the two *arsC* alleles matched what was observed with the gel after electrophoresis.

qPCR was next used to determine the copy number of *arsC* in AF293. The C_T values were determined for the *arsC* sequences and compared to those of *FKS1*, which was used as the single-copy control. The output graph from the reaction shows an earlier C_T for *arsC* than for *FKS1* (Fig. 2), consistent with the greater copy number of *arsC*. Calculation of the copy number of *arsC* for AF293 and for other isolates demonstrated that *arsC* is present in two copies (Table 4), which confirmed that our strategy could determine the copy number of multicopy sequences.

Determination of rDNA copy number. Once *FKS1* was established as a reliable single-copy control, this sequence was used to determine the copy numbers of the rDNA genes in *A. fumigatus* by quantitating the copy number of the 18S rDNA subunit. Since the copy number of the rDNA genes of AF293 has been determined from the genome sequence, this isolate was used in a pilot TaqMan assay in which *FKS1* (single copy) was used to calculate the copy number of the 18S rDNA subunit. According to the genome sequence, AF293 has 35 copies of the rDNA genes per genome (34). Figure 3 shows an example of the plots of *FKS1* versus 18S rDNA and clearly demonstrates that there are more copies of the 18S rDNA gene than the *FKS1* gene. Calculation of the 18S rDNA copy numbers resulted in a value of 38 copies of 18S rDNA per genome in AF293, which is in fairly close agreement with the genomic copy number (38 versus 35 copies) for AF293 (34). The 18S rDNA copy numbers of the remaining isolates were then determined using *FKS1* as the reference gene. The data show a range of 38 to 91 copies, with an average of 54 copies per genome (Table 5). These results show that for our set of isolates, 18S rDNA copy numbers are isolate specific and can vary substantially from strain to strain.

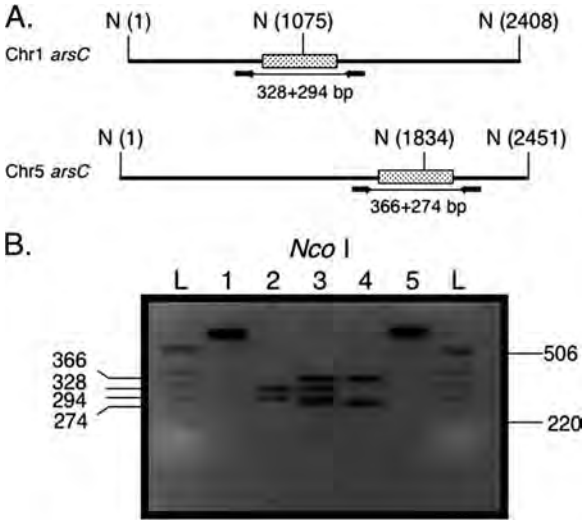

FIG. 1. Confirmation of *arsC* copy number in AF293. (A) Priming sites for the two *arsC* alleles. The Chr1 *arsC* allele is located on chromosome 1, while the Chr5 *arsC* allele is located on chromosome 5. Primers are indicated by black arrows; PCR product is indicated by the line connecting the primers. The three NcoI sites (N) (one located within and two flanking the *arsC* genes), with locations given as base pairs, are indicated within parentheses. Stippled boxes are the *arsC* open reading frames. The predicted sizes of the fragments after NcoI digestion are indicated below each open reading frame. (B) NcoI digestion of *arsC* PCR products. Lane 1, uncut Chr1 *arsC* PCR product; lane 2, NcoI digest of Chr1 *arsC*; lane 3, mixture of both NcoI digestions; lane 4, NcoI digestion of Chr5 *arsC* PCR product; lane 5, uncut Chr5 *arsC* PCR product. Sizes are in base pairs. L, ladder. Ladder sizes are at the right of the gel; fragment sizes are at the left.

Stability of rDNA copy number. Since our results indicated that rDNA copy numbers could vary among strains of *A. fumigatus*, we investigated various environmental conditions to determine whether or not an effect on copy number could be observed. Factors that were investigated included morphology, growth temperature, culture age, antifungal exposure (itraconazole), and animal model organ site (lung versus kidney). The overall copy number mean was found to be 38.032 ± 0.13, which agrees with our initial copy number determination for AF293. However, Table 6 shows that some significant differences in copy numbers were observed among our growth conditions (morphology, growth temperature, and culture age). In spite of these differences, variation in copy numbers among all conditions tested was less than 1 copy and the copy numbers would all have been 38 copies if numbers were rounded.

DISCUSSION

Timely diagnosis of IA is challenging due to the lack of specific clinical manifestations of infection. Unfortunately, symptoms can be nonspecific and include fever, cough, dyspnea, chest pain, and apnea. Therefore, diagnosis can be dependent on the combination of a strong index of suspicion and radiologic findings, serologic assays, or when possible, culture and/or histologic findings (24). For any of these methods, a quantitative estimate of fungal burden is difficult at best and can be expensive or time-consuming. In fact, even under controlled experimental conditions of animal modeling, colony counts can be misleading, as some studies have noted decreasing counts that are contradicted by other measurements with the same animal (36, 37). The ubiquitous nature of *A. fumiga-*

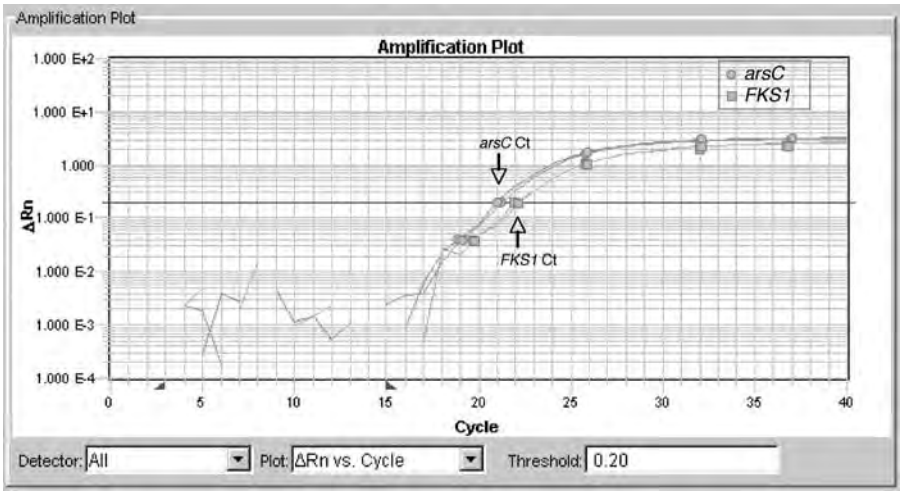

FIG. 2. Amplification plot of AF293 *arsC* versus *FKS1* TaqMan assays. TaqMan assays were performed using an *arsC* primer-probe combination and *FKS1* primer-probe combination. The graph represents a sample plot from duplicate reactions run on aliquots of the same DNA sample. Amplification of the *arsC* gene is denoted by circles. Amplification of the *FKS1* gene is denoted by squares. The C_T value of the *arsC* line is approximately 21.1 (downward arrow), and the C_T value of *FKS1* is approximately 22.1 (upward arrow).

tus in the environment and associated possibility of inhaling fungal elements that may or may not grow in vivo, but could be detected as CFU after lavage, further complicate making an accurate assessment.

Through advances in instrumentation and reagent chemistry, PCR continues to find new applications in clinically relevant areas. In spite of not being widely employed as a routine clinical diagnostic tool for detecting IA, PCR is proving increasingly useful as an investigational tool for studying aspergillosis both in vitro and in vivo and may ultimately find its way into the clinical laboratory as a routine diagnostic tool for IA. For in vivo applications of animal infections, qPCR is often used to make a determination of the number of CFU, which are frequently expressed as conidial equivalents in order to indicate one nucleus per conidium. While the number of CFU is fairly accurate for fungi that grow in a yeast morphology, the number of CFU obtained by plate counts can be difficult to interpret for filamentous organisms due to the inability to distinguish a single hypha that forms one colony from the same fragmented hypha that yields multiple colonies. In fact, using CFU for measuring *A. fumigatus* fungal loads has been shown to yield equivocal results (6, 36). Therefore, alternative methods that do not require obtaining viable colony counts but provide some indication of fungal burden are potentially useful for quantifying the fungal load of a given specimen. qRT-PCR is exceptionally well suited for this requirement. In fact, when all protocols are standardized, from infection model through tissue preparation, reproducible results can be obtained, even among interlaboratory studies (35).

TABLE 4. Determination of *arsC* copy number of all isolates by qRT-PCR in comparison to that of *FKS1*[a]

Strain	*FKS1* avg C_T	*FKS1* copy no.	*arsC* avg C_T	*arsC* copy no.[b]
AF293	22.1 ± .107	1	21.1 ± .007	2 (2.00)
WSA-172	19.8 ± .010	1	18.7 ± .004	2 (2.14)
WSA-446	22.0 ± .052	1	21.1 ± .003	2 (1.89)
WSA-445	19.8 ± .014	1	18.7 ± .005	2 (2.16)
WSA-271	22.1 ± .025	1	21.0 ± .002	2 (2.07)
WSA-270	19.2 ± .042	1	18.2 ± .002	2 (2.00)
WSA-621	19.9 ± .060	1	18.8 ± .003	2 (2.01)
WSA-419	23.1 ± .014	1	21.9 ± .007	2 (2.42)

[a] C_T values are means ± standard deviations (three samples, run in duplicate).
[b] *arsC* copy numbers were determined using the formula $2^{-\Delta\Delta CT}$. Results rounded to whole numbers are shown, with the unrounded results given in parentheses.

The observations in this study add an important caveat for standardized procedures to now include working with the same *A. fumigatus* strain when qPCR quantitation using the rDNA genes is required. Our results have shown that using an 18S rDNA target requires prior knowledge of copy number of the strain of interest. With our small sample size, we found copy numbers to vary by as much as ~2.5×. Neither the upper limit nor the lower limit of 18S rDNA copy number is known, but it is almost certain to vary by a larger amount than the amount that we observed for our set of isolates. Consequently, 18S rDNA copy number cannot be assumed based on another value previously determined from an unrelated strain. This observation presently does not have direct clinical implications, since qPCR is not routinely used to diagnose IA and fungal burden is rarely part of any diagnosis, since for at-risk patients a positive assay regardless of amount is always cause for concern. However, accurate quantitation of *A. fumigatus* CFU has numerous applications, many of which have clinically relevant consequences. These include data generated from more than one strain or testing unknown strains in experiments measuring tissue burdens, in vivo drug susceptibility testing, environmental quantitation, tracking CFU during disease progression, or comparison of different methods for measuring fungal load (2, 17, 32, 36, 42). Similarly, direct quantitative comparisons of the same or different strains that utilize qPCR versus some other method, such as CFU counts or galactomannan detection, can be erroneous in the absence of an accurate rDNA copy number. Finally, model systems that may use the same assay but different strains and report results in

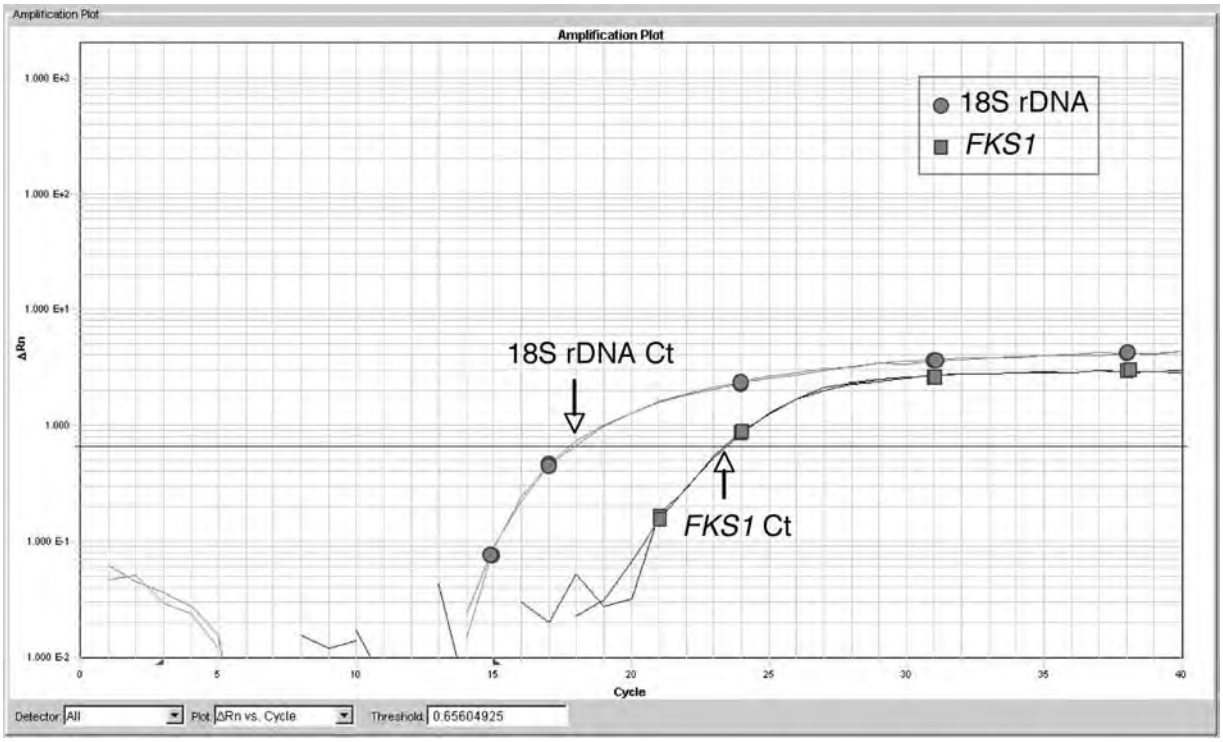

FIG. 3. Amplification plot of 18S rDNA versus *FKS1*. An example of copy number determination of 18S rDNA using *FKS1* as a single-copy control. The figure is an amplification plot of a TaqMan assay performed using the 18S rDNA primer-probe combination and *FKS1* primer-probe combination. Template DNA was taken from the same DNA sample prepared from AF293 and run in duplicate. Note the earlier C_T value of 18S rDNA (circles), which is approximately 18.0 (downward arrow) versus the *FKS1* C_T value (squares), which is approximately 23.4 (upward arrow). The lower C_T value for 18S rDNA reflects the greater copy number of the target, since the fluorescence crosses the threshold at a much lower cycle number.

CFU, such as animal survival studies, typically use absolute numbers and therefore need to be calculated accurately if qPCR is part of the methodology. However, in spite of the variation between strains, our results suggest that within-strain variation, at least in the case of AF293, is negligible. Therefore, in studies that utilize the same strain and involve quantitation, qPCR using the rDNA genes should yield consistent results. We could not identify any condition that was able to cause the 18S subunit number to vary by more than 1 copy within AF293, in spite of investigating a number of stress conditions. However, we did identify some significant differences in our analyses. We suspect these differences may have been due to experimental error since qPCR accuracy requires precise technique. On the other hand, we know nothing about the

TABLE 5. *A. fumigatus* 18S rDNA copy number determinations

Strain	No. of 18S rDNA copies[a]
AF293	38 ± 0.01
WSA-172	46 ± 0.03
WSA-446	47 ± 0.01
WSA-445	49 ± 0.06
WSA-271	49 ± 0.05
WSA-270	53 ± 0.01
WSA-621	70 ± 0.03
WSA-419	91 ± 0.03

[a] Values are mean ± standard deviations (three samples, run in duplicate).

TABLE 6. *A. fumigatus* 18S rDNA copy number stability

Condition	Subgroup	No. of 18S rDNA copies[a]	*P* value
Morphology	Conidia	38.02 ± 0.011	0.03
	Hyphae	38.11 ± 0.01	0.03
Temperature	30°C	37.84 ± 0.044	0.03
	45°C	38.03 ± 0.015	0.03
Culture age	3 days	38.041 ± 0.024	0.004
	5 days	37.906 ± 0.059	0.004
	10 days	38.321 ± 0.019	0.004
	25 days	38.061 ± 0.017	0.004
Itraconazole concn	0.00 μg/ml	37.984 ± 0.049	0.17
	0.03 μg/ml	38.024 ± 0.015	0.17
	0.06 μg/ml	38.039 ± 0.022	0.17
	0.125 μg/ml	38.033 ± 0.012	0.17
	0.25 μg/ml	38.001 ± 0.055	0.17
	0.5 μg/ml	38.046 ± 0.009	0.17
	1.0 μg/ml	38.039 ± 0.019	0.17
	2.0 μg/ml	38.034 ± 0.038	0.17
Mouse	Lung	38.056 ± 0.038	0.13
	Kidney	38.136 ± 0.008	0.13
Guinea pig	Lung	38.231 ± 0.008	0.13
	Kidney	37.688 ± 0.059	0.13

[a] Copy values are means ± standard deviations.

mechanism by which copy number variation occurs and what, if any, phenotypic consequences are associated with changes in copy number within a strain. The fact that different strains of *A. fumigatus* have different rDNA copy numbers is evidence that variation occurs. Since our qPCR assay can detect only whole copies (a fraction of a copy would not yield a PCR product), the data could have arguably been rounded to the nearest whole copy. In this case, all copy numbers would round to 38, which matches the control AF293 number. However, since we cannot rule out copy number heterogeneity within a population, we chose not to round the data. Future studies of copy number should focus on whether changes are rapid, such as by an unequal recombination event that leads to large gains or losses of rDNA repeats, or gradual, which could result in small changes of a unit or two over longer periods of time. Understanding the mechanism may reveal whether or not the changes are responses to selection or are random, without clear phenotypic consequences.

In spite of the observed copy number variation within *A. fumigatus*, application of these results to other species of *Aspergillus* probably should not be done without empirical analysis. *Aspergillus* taxonomy can be complicated by the existence of sections, which may not be discriminated at the clinical level but can be discriminated at the molecular level. For example, in the *Aspergillus* section *Fumigati*, *A. fumigatus* may not be discriminated from other members, such as *A. lentulus* or *A. brevipes*. However, these species can be identified by sequencing select loci (i.e., β-tubulin). Therefore, rDNA variation could possibly indicate a separate subspecies. In our study, we confirmed that our strains were all *A. fumigatus* using β-tubulin sequencing (data not shown), but since so little is known at the molecular level about these subgenera, confirmational sequencing of additional loci may be required when trying to quantitate unknown isolates.

Although we targeted the 18S rDNA subunit in this study, determination of copy number should hold for targets that lie within the 28S subunit or between the two subunits (ITS1, ITS2, and 5.8S) as well, since the large and small ribosomal subunits, though multicopy and tandemly arrayed, are colinear and transcribed as a single transcript along with the intervening ITS region (21, 43). Therefore, based on what is known in model fungi, the copy numbers of the 18S and 28S genes, as well as the intervening sequences, should be the same in the same strain of *A. fumigatus*. The advantage of primer design in the more variable ITS1, ITS2, or even the D1/D2 region of the 28S subunit is that species specificity can be possible, subspecies issues as described above notwithstanding. If, on the other hand, the increased sensitivity of targeting the multicopy rDNA genes is not needed, a suitable single-copy gene (i.e., *FKS1*, *ARG4*, or *pyrG*) can be used with fairly high confidence that its copy number will be invariant among unrelated strains and equal to 1. Finally for presence or absence outcomes, copy number variation is probably not a concern; however, given that the ribosomal genes are usually targeted due to their increased sensitivity, if investigators are quantitating cell numbers using these genes, the strain-specific variability of rDNA copy number may be an important factor that affects the sensitivity of PCR assays for quantifying *Aspergillus fumigatus*.

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Interlaboratory Reproducibility of a Single-Locus Sequence-Based Method for Strain Typing of *Aspergillus fumigatus*▽

S. F. Hurst,[1] S. E. Kidd,[2] C. O. Morrissey,[2] E. Snelders,[3] W. J. G. Melchers,[3] M. V. Castelli,[4] E. Mellado,[4] K. Simmon,[5] C. A. Petti,[5] S. Richardson,[6] S. Zhang,[6] A. M. Romanelli,[7] B. L. Wickes,[7] H. A. de Valk,[8] C. H. W. Klaassen,[8] and S. Arunmozhi Balajee[1]*

Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, Georgia[1]; Department of Medicine, Monash University, Central and Eastern Clinical School, Alfred Hospital, Melbourne, VIC, Australia[2]; Department of Medical Microbiology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands[3]; MadSpain Servicio de Micologia, Centro Nacional de Microbiologia Instituto de Salud Carlos IIrid, Majadahonda, Madrid, Spain[4]; ARUP Laboratories, Salt Lake City, Utah[5]; Ontario Ministry of Health, Public Health Laboratories Branch, Toronto, Canada[6]; the University of Texas Health Science Center at San Antonio, San Antonio, Texas[7]; and Canisius Wilhelmina Hospital, Nijmegen, The Netherlands[8]

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Seven international laboratories tested the recently proposed single-locus typing strategy for *Aspergillus fumigatus* subtyping for interlaboratory reproducibility. Comparative sequence analyses of portions of the locus AFUA_3G08990, encoding a putative cell surface protein (denoted CSP), was performed with a panel of *Aspergillus* isolates. Each laboratory followed very different protocols for extraction of DNA, PCR, and sequencing. Results revealed that the CSP typing method was a reproducible and portable strain typing method.

Strain typing of *Aspergillus fumigatus* can be important for detecting outbreaks and in epidemiological investigations. Recently a novel, simple, and rapid single-locus sequence typing strategy was proposed as a typing tool for *A. fumigatus* (2). Genetic diversity in this locus arises from both tandem repeats and point mutations of the gene encoding the putative cell surface protein (CSP), AFUA_3G08990 (2). Balajee et al. employed this method (denoted CSP typing) to subtype 55 epidemiologically linked *A. fumigatus* isolates obtained from six nosocomial outbreaks of invasive aspergillosis and found the technique satisfied the tenets of a good subtyping method (6), since it identified distinct genotypes as well as clusters of closely related isolates (clonal complex). Although a subsequent study found that CSP typing had lesser discriminatory power than a microsatellite-based method, CSP typing remains useful as a quick frontline strategy for *A. fumigatus* strain discrimination (1). Importantly, since CSP typing employs a comparative sequencing strategy, it does not require elaborate training or software for analyses and is relatively user-friendly and economical and therefore amenable for use in reference microbiology laboratories. Other available subtyping methods, such as microsatellite (e.g., Str*Af*)-based assays) (3) and *Afut1* DNA hybridization profiles (*Afut1* method) (4), have superior discriminatory power but need specialized equipment and dedicated software. Also, since reproducibility studies have not been conducted using these techniques, the data obtained cannot be readily shared between laboratories.

Balajee et al. evaluated the CSP typing method for typeability, in vitro stability, intralaboratory reproducibility, and concordance with other typing methods (2). However, the interlaboratory reproducibility of this method has not been tested so far. Given that one of the hallmarks of a good typing method is reproducibility which is independent of the operator, place, and time (5), we examined the reproducibility of CSP typing in diverse laboratory settings with data generated under a wide array of experimental conditions.

To test interlaboratory comparability, a panel of *A. fumigatus* isolates was selected from outbreak isolates whose CSP genotypes were established in a previous study (1, 2). In brief, *A. fumigatus* isolates used in this study were obtained from previous cases in invasive aspergillosis outbreaks and represented both clonal and distinct genotypes (as verified by the CSP typing, *Afut1*, and STR*Af* methods [1, 2]). Species identification of all *A. fumigatus* isolates was confirmed by sequence comparison of the β-tubulin region (2).

The panel consisted of 14 *A. fumigatus* isolates: 5 isolates shared the same CSP type (arbitrarily designated genotype 1), 8 isolates shared another CSP type (genotype 2), and 1 isolate had a unique CSP type (genotype 3). In addition, one isolate of *Aspergillus flavus* (CDC 14) was included as an outlier. Isolates were randomly coded, subcultured on Sabouraud dextrose agar slants, and then sent to seven international laboratories, which represented research, clinical, and reference facilities. Each laboratory was also provided with the following *A. fumigatus*-specific primers: 5′-TTGGGTGGCATTGTGCCAA (forward) and 5′-GGAGGAACAGTGCTGTTGGTGA (reverse). These primers amplify a ~550- to ~700-bp fragment of the AFUA_3G08990 gene (dependent on the number of repeats). The participating laboratories cultured, isolated DNA, and performed PCR, sequencing, and DNA sequence analysis using their own routine methods.

The participating laboratories were requested to do the fol-

* Corresponding author. Mailing address: Mycotic Diseases Branch, Centers for Disease Control and Prevention, Mail Stop G 11, 1600 Clifton Road, Atlanta, GA 30333. Phone: (404) 639-3337. Fax: (404) 639-3546. E-mail: fir3@cdc.gov.

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lowing: (i) generate CSP sequences from the panel of isolates and align the sequences using the Af293 (*A. fumigatus* isolate whose genome has been completely sequenced) CSP sequence as a reference (GenBank accession no. XM_749624); (ii) visually identify unique and shared genotypes, (iii) assign arbitrary designations to each distinct CSP genotype represented by one or more isolates in the panel—for example, if isolates 1, 2, and 3 were observed to have related genotypes, they were assigned to genotype X; (iv) submit the arbitrary genotype assignments and all sequences in FASTA format via e-mail to the coordinating laboratory; and (v) send detailed protocols on the methods used to generate the sequences to the coordinating laboratory. Each participating laboratory cultured, isolated DNA, and performed PCR, sequencing, and DNA sequence analysis using methods which were routine in their individual laboratories.

Culture methods included seven different media (both broth and agar based) and two incubation temperatures (30°C and 37°C). For DNA extraction, two laboratories harvested mycelial mats and five harvested mycelia and spores from plates. One laboratory collected only spores for DNA isolation. DNA isolation utilized a variety of methods, including commercially available kits and in-house protocols. Only one laboratory quantitated the isolated genomic DNA and made working dilutions of equal concentrations (10 ng/μl), while the others used the genomic DNA directly in the PCR, regardless of concentration.

PCR cycling was carried out on four models of thermal cyclers from three manufacturers. PCR amplifications were accomplished with either commercially available kits (one laboratory) or in-house PCR mixes (six laboratories), utilizing four different polymerases. All laboratories visualized the PCR products on an agarose gel, either commercial or made in-house. PCR product cleanup was performed either by use of the ExoSap enzyme reaction (one laboratory), use of magnetic beads (one laboratory), or column purification (five laboratories). Five laboratories estimated the concentrations of purified PCR products by comparison to a commercial mass ladder standard on an agarose gel, while two laboratories utilized the NanoDrop UV reader (Thermo Scientific) to quantitate the PCR products. One laboratory used the PCR products regardless of the concentration. Sequencing was performed using either Applied Biosystems BD 3.1 or BD 1.1 Dye Terminator chemistry or DYEnamic ET Dye terminator chemistry (GE Healthcare) on three different models of capillary electrophoretic sequencers. One laboratory utilized a commercial sequencing service. All laboratories sequenced both the forward and reverse strands, and sequence editing was performed using the Sequence Analyzer, Contig Express, MacVector, Sequencher, or BioEdit software package. Sequence alignments were assembled using the BioEdit 7.0.9, ClustalX 1.83, Lasergene 8.0, or Mega 4.0 software program.

Despite the wide spectrum of reagents, equipment, and methods used to obtain the CSP sequences, five laboratories assigned the correct genotype to all isolates, yielding 100% concordance (Table 1), while laboratories 5 and 6 reported a concordance of only 93% and 85%, respectively. Laboratory 5 reported the sequence from isolate CDC 3 as genotype 1, when the correct designation for this isolate was genotype 2. Similarly, laboratory 6 identified the isolate CDC6 as genotype 2, when the correct designation should have been genotype 3. The sequences obtained by these laboratories were of high quality and were identical to those of the genotypes that were incorrectly assigned. All other sequences generated by these laboratories were also of high quality with no base-call errors. Therefore, we speculate that laboratories 5 and 6 may have reported incorrect genotype designations because of possible cross-contamination with another isolate from the *Aspergillus* panel. Alternatively, this could be also be attributed to an inadvertent exchange of samples that may have occurred at any stage of the process from culturing of the organism to DNA extraction to PCR or sequencing. Six laboratories reported that isolate CDC14 yielded no PCR product; this was expected, since this isolate was *A. flavus* and should not be amplifiable with the primer set provided. Laboratory 6 reported this isolate as belonging to genotype 1, reiterating the likelihood of contamination problems in this laboratory.

TABLE 1. CSP genotypes assigned to the *Aspergillus* panel, as reported by participating laboratories

Isolate	Genotype reported by[a]:						
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
CDC 1	2	2	2	2	2	2	2
CDC 3	2	2	2	2	1*	2	2
CDC 4	1	1	1	1	1	1	1
CDC 5	2	2	2	2	2	2	2
CDC 6	3	3	3	3	3	2*	3
CDC 7	2	2	2	2	2	2	2
CDC 8	1	1	1	1	1	1	1
CDC 9	1	1	1	1	1	1	1
CDC 10	1	1	1	1	1	1	1
CDC 12	2	2	2	2	2	2	2
CDC 14	NP	NP	NP	NP	NP	1*	NP
CDC 15	2	2	2	2	2	2	2
CDC 19	2	2	2	2	2	2	2
CDC 20	2	2	2	2	2	2	2
CDC 21	1	1	1	1	1	1	1

[a] Each of the laboratories (labs) assigned a genotype number to all *A. fumigatus* isolates (except CDC 14, which is *A. flavus*). NP, no product; *, incorrectly assigned genotype.

The participating laboratories aligned the sequences and assigned genotype scores by visual inspection as described previously (2). The number of isolates in the panel was relatively small, and the differences in repeat number are easy to see in aligned sequences. However, this type of visual analysis would be difficult in larger studies, and a more robust, objective genotype scoring system, which would remove any potential for human error in genotype assignment, should be developed for such analyses. Interestingly, the limiting factor of this typing strategy was strain contamination and/or human error involving sample exchange, rather than sequencing errors or subjective data interpretation. *Aspergillus* spores are easily aerosolized, and extreme care must be taken when working with these organisms to prevent contamination. Assuming that appropriate precautions are taken to prevent contamination, we demonstrate here that CSP typing performed in different laboratories was concordant and results can therefore be compared directly, despite considerable variation in protocols.

Recently the STR*Af* method was demonstrated to have good interlaboratory reproducibility for *A. fumigatus* subtyping (4). In this study, where five laboratories participated, nonspecific

amplification products, bleed-through of the different fluorescent labels, and inexperience of laboratories led to some inconsistencies in results. Here, we present results of another multicenter study for *A. fumigatus* subtyping that also had superior reproducibility. Such multilaboratory reproducibility studies are essential to ensure that any proposed subtyping method can be reliably employed for epidemiological studies.

Additionally and importantly, all data in this study were shared via the Internet, thus confirming that the CSP typing scheme can be a portable and thereby convenient strategy for interlaboratory data sharing or comparison. Furthermore, the data from such studies can easily be stored in a database and archived, retrieved, and reanalyzed at any time, making this a useful tool for global molecular epidemiological investigations of *A. fumigatus*. The use of inexpensive or free Web-based software for data analysis makes this an attractive tool for small or cost-conscious laboratories. In summary, this international, multilaboratory study confirms the reproducibility and portability of the CSP typing method.

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Case Report

A case of bovine valve endocarditis caused by *Engyodontium album*

SHIVA BALASINGHAM*, SPYROS CHALKIAS*, ANNA BALASINGHAM*, ZANE SAUL†, BRIANL .WICKES‡ & DEANNA A. SUTTON§

**Bridgeport Hospital/Yale University Internal Medicine Program, Bridgeport, Connecticut, †Bridgeport Hospital, Department of Infectious Disease, Bridgeport, Connecticut, ‡Department of Microbiology and Immunology, and §DepartmentofP athology, Universityof TexasH ealthSci enceC enterat San Antonio,San Antonio,Texas,USA*

We report the first case of *Engyodontium album* bioprosthetic valve endocarditis in a 44-year-old male with a history of juvenile rheumatoid arthritis. There is only one other report of *Engyodontium album* as a human pathogen. The present case supports the increased incidence of fungal endocarditis especially in patients receiving immunotherapy.

Keywords *Engyodontium album*, fungale ndocarditis

Introduction

Although rare, fungal endocarditis is a severe disease that requires long-term treatment and often surgical intervention [1–3]. Recent case reports on disseminated fungal infections have involved uncommon etiologic agents that had not been previously identified as human pathogens. This seems to be particularly the situation with patients receiving chemotherapy or immunotherapy [4,5]. In 1990, Augustinsky *et al*. reported the first case of *Engyodontium album* endocarditis of a native aortic valve [6]. We present the first case of *Engyodontium album* endocarditis involving a bovine mitral valve of a patient receiving metho-trexate for juvenile rheumatoid arthritis. This adds *Engyodontium album* to the spectrum of opportunistic fungi that may cause human infections involving bioprosthetic material.

Caser eport

The patient is a 44-year-old male with a medical history of juvenile rheumatoid arthritis who was receiving methotrexate and prednisone therapy. In 2008, he developed *Streptococcus viridians* endocarditis with discitis, involving both the mitral and tricuspid valves, that was so extensive that he promptly underwent surgery. The mitral valve was replaced with a 31 mm bovine pericardial thermafix bioprosthesis, while the tricuspid valve required debridement of vegetations, excision of the posterior cusp and cords, and annuloplasty. Upon completion of a 4-week course of parenteral ceftriaxone he was found to have 3rd degree heart block which required the implantation of a dual-chamber pacemaker (Medtronic 5076: Medtronic Inc., Minneapolis, MN).

In February 2010, the patient was admitted for recurrent episodes of slurred speech and fever. A CT scan of the head at the time did not reveal any acute abnormalities. However, a transesophageal echocardiogram showed a 2 × 2cm vegetation on the posterior leaflet of the bioprosthetic mitral valve. No vegetations were visualized on the other three valves. The bioprosthetic valve was subsequently replaced with a 31mm St. Jude mechanical valve (St. Jude Medical, St. Paul, MN).

On gross pathology, the bioprosthetic mitral valve had an irregular surface with friable tissue that nearly completely obstructed its lumen but the cusps were intact (Fig. 1A and B). Histopathology of the vegetation revealed abundant fungal organisms and necrotic tissue on Gram's stain (not shown), the hematoxylin and eosin stain (Fig. 2A), and the Gomori's methenamine silver stain (Fig. 2B).

Material obtained from the vegetation was submitted to the microbiology laboratory for culture and identification. Initially, the fungus was identified as an *Acremonium* species and later as a *Beauveria* species. The patient was treated with parenteral liposomal amphotericin B (250 mg daily) and oral flucytosine (170 mg four times

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Correspondence: Shiva Balasingham, 36 Myrtle Avenue, Bridgeport, CT 06604, USA. Tel: +1 203 520 5393; fax: +1 203 384 4294; Email: shiva.balasingham@gmail.com

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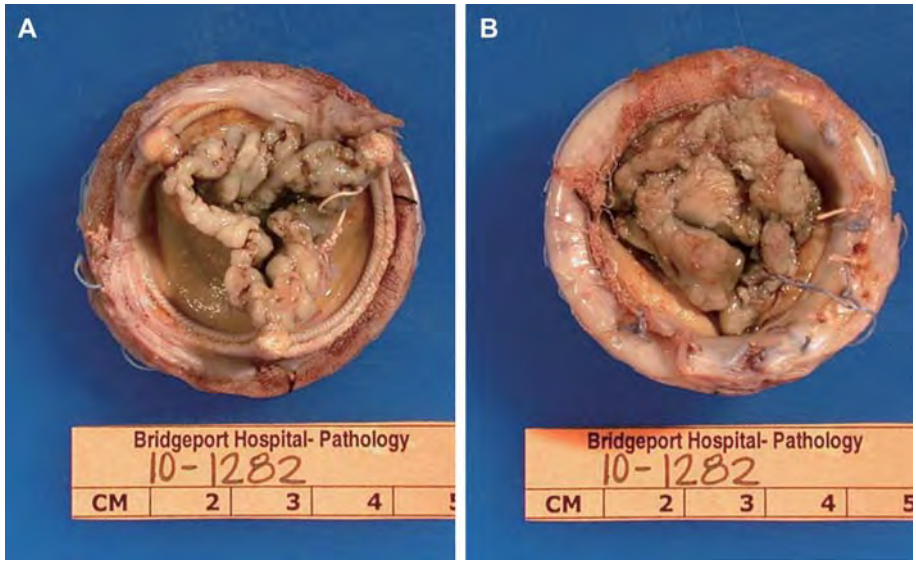

Fig. 1 Gross pathology of the bioprosthetic mitral valve. (A) Inferior aspect, and (B) superior aspect.

daily). The amphotericin B was discontinued due to acute kidney injury and the patient was switched to parenteral voriconazole (280 mg twice daily). After 10 days, the patient was discharged home where he completed a 6-week course of oral voriconazole (200 mg twice daily).

Morphological identification

Because this fungus was identified as a *Beauveria* species, a genus which has not previously been reported as an etiologic agent of endocarditis, it was decided to determine the species and obtain antifungal susceptibilities. The fungal isolate was forwarded to the Fungus Testing Laboratory at the University of Texas Health Science Center in San Antonio for further identification and susceptibility testing, and accessioned into their culture collection as UTHSC 10-669.

The fungal isolate was subcultured onto potato flakes agar (PFA) and carnation leaf agar (CLA) plates, both prepared in-house, and incubated at 25°C. A 25°C slide culture was also prepared on PFA and examined after 5 days incubation.

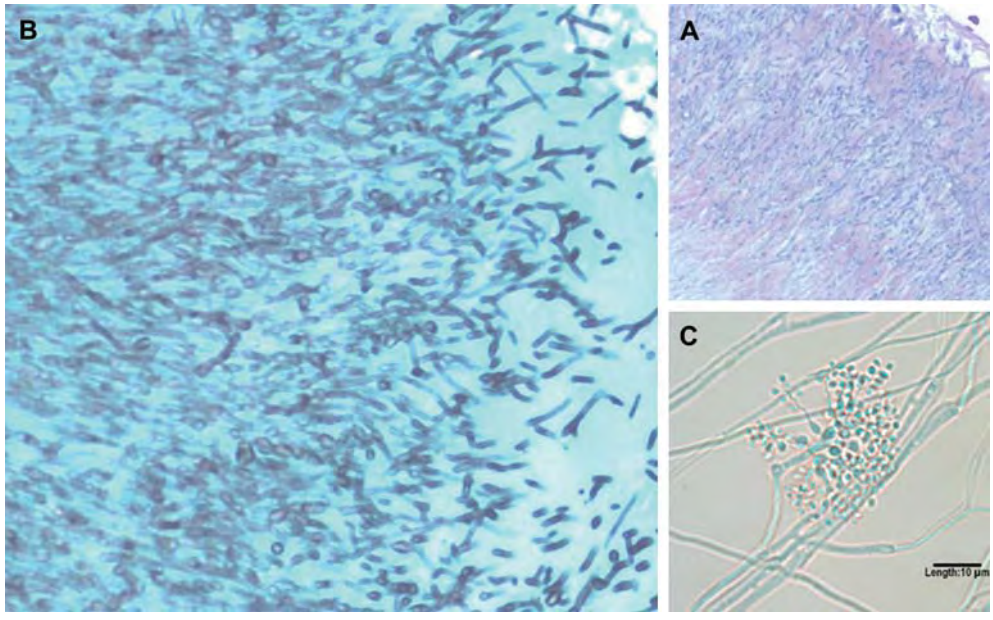

Fig. 2 (A) Hematoxylin and eosin stain of vegetation on the valve tissue. (B) Gomori methenamine silver stain of the vegetation under higher magnification. (C) Slide culture preparation of *Beauveria bassiana* on potato flakes agar after 7 days incubation at 25°C demonstrating clusters of conidiogenous cells along discrete areas of the hyphae not seen in *Engyodontium album*.

Growth on PFA was moderately rapid with colonies that were white to slightly cream-colored, lanose to floccose and expanding without any discrete areas of conidiogenesis typically seen with the members of the genus *Beauveria* (Fig. 2C).

Mycelial growth was more restricted on CLA, with agar surface displaying a pale, thin, translucent lawn of hyphae and conidiogenous cells in whorls. These whorls consisted of up to four conidiogenous cells ranging from 15–30 μm long, and terminated in a geniculate (or zigzag) rachis bearing smooth, hyaline, subglobose and mostly apiculate (pointed at the base) conidia (approximately 1.5 × 2 μm) on delicate denticles (Fig. 3A). A photomicrograph of the isolate taken with a Joel JSM-6610LV scanning electron microscope, sputter-coated with gold pallidum, clearly demonstrates the method of conidial production (Fig. 3B).

Temperature studies also demonstrated growth of the isolate at 35°C. Based upon the macroscopic, microscopic, and physiologic features cited above, the organism was identified phenotypically as *Engyodontium album*. The isolate has been deposited into the University of Alberta Microfungus Collection and Herbarium under the accession number UAMH 11234. Further molecular characterization of the isolate was performed under the accession number UTHSC R-4523.

Molecularide ntification

The isolate was prepared for sequencing by growing on potato dextrose agar for 20 h at 30°C. Template DNA was recovered as described using the Prepman Ultra reagent (Applied Biosystems, Foster City, Ca) [7]. Template DNA was then amplified using the ITS-1 as a forward primer and NL-4 as a reverse primer [8] using previously described conditions [7]. PCR products were purified using the Qiaquick PCR purification kit and sequenced at the University of Texas Health Science Center at San Antonio Advanced Nucleic Acids Core Facility using the ITS-1, ITS-4, NL-1 and NL-4 as sequencing primers as described [7].

The ITS and D1/D2 sequences were then used to perform a BLASTn search of the Genbank database (http://blast.ncbi.nlm.nih.hov/Blast.cgi). Search results were sorted on maximum percent identity and the top three records with >90% query coverage were evaluated for relatedness to R-4523. The top three ITS results were all at 100% identity and consisted of *Engyodontium* species (accession # GQ221128), *Engyodontium album* (accession # DQ649066), and *Engyodontium album* (accession # AB106650). The top three D1/D2 results were *Verticillium* species (accession # AY312607) with 99% identity, *Engyodontium album* (accession # AF049167) with 99% identity, and *Engyodontium album* (accession # DQ872372) with 98% identity. Based on these results we concluded that R-4523 was most closely related to *Engyodontium album*.

The sequence was deposited in Genbank under the accession # HM214541 and D1/D2 sequence was deposited under the accession # HM214540.

Antifungal susceptibility testing

In vitro antifungal susceptibility testing of the isolate was assessed using the previously published Clinical and Laboratory Standards Institute (CLSI) document M38-A2 for testing of filamentous fungi. Briefly, the testing parameters included a final inoculum of 0.4×10^4 to 5×10^4 CFU/mL consisting of conidia and hyphal fragments standardized spectrophotometrically, 35°C incubation, and the use of RPMI-1640 medium (Hardy Diagnostics, Santa Maria, CA) for all agents except amphotericin B (AMB, Bristol-Myers Squibb, New York, NY) which was tested in antibiotic medium 3 (Difco, a Division of Becton Dickson, Sparks, MD). Drug concentrations ranged from 0.03–16 μg/ml for AMB, itraconazole, ketoconazole (ITZ, KTZ, Janssen Pharmaceutica, Division of Janssen-Ortho, Inc., New Brunswick, NJ), and voriconazole (VRZ, Pfizer, Inc., New York, NY). Concentrations of fluconazole (FLZ, Pfizer Inc., New York, NY) ranged from 0.125–64 μg/mL and from 0.015–8 μg/mL for anidulafungin (ANID, Pfizer Inc., New York, NY).

Tests were incubated at 35°C until growth was noted in the growth control. Minimum inhibitory concentrations (MIC) were defined as the lowest concentration resulting in complete inhibition of growth for AMB, ITZ, and VRZ, and 50% inhibition for KTZ and FLZ. The minimum effective concentration (MEC) read for ANID was defined as the point where a noticeable change in the growth characteristics was noted. MIC's/MEC's read at 72 and 96 h are shown in Table 1.

Discussion

Fungal species are uncommon agents of endocarditis, involved in only 2–4% of all cases [3]. The mean age of patients at presentation is typically around 44 years [9]. The primary risk factors include injection drug use, prosthetic heart valves, and immunocompromised status [3]. The most common pathogens are *Candida* spp. and *Aspergillus* spp., with all other cases being caused by pathogens rarely encountered in the clinical laboratory [3]. Our patient had a history of mitral valve replacement with a bioprosthetic valve and was on long-term corticosteroid therapy and methotrexate for juvenile rheumatoid arthritis, putting him at risk for developing fungal endocarditis.

In the era of advanced chemotherapy and disease modifying antirheumatic agents, an increasing incidence of opportunistic mycoses has been recorded [10]. Cellular immune response impairment due to the use of antibodies

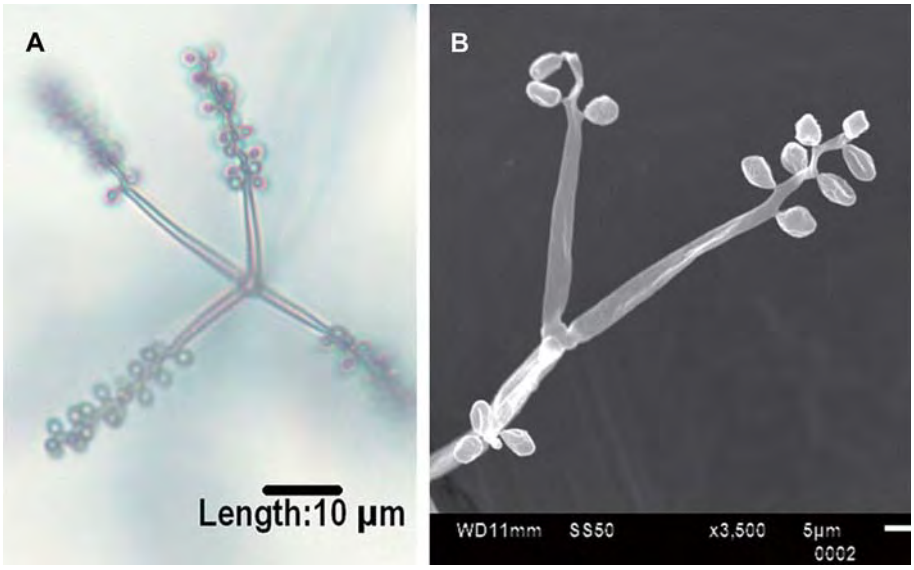

Fig. 3 (A) Slide culture preparation of *Engyodontium album* on potato flakes agar after 7 days incubation at 25°C demonstrating geniculate conidiogenous cells in whorls and conidia. (B) Scanning electron microscopy of *Engyodontium album* clearly demonstrating method of conidial production.

against tumor necrosis factor (TNF), as well as drugs such as methotrexate has been related to several cases of opportunistic infections in humans [11]. These fungi may cause disseminated and fatal infections in the human host whose defenses have been impaired by such drugs. It is proposed that fungi, which are ubiquitous in nature, gain entry into the host through skin trauma or via the respiratory tract. Once inside the host they may disseminate hematogenously via active conidiogenesis from their initial site of inoculation, thus involving internal organs and tissues. This has been observed in cases of *Acremonium strictum* infection, and is the proposed mechanism of dissemination for *Beauveria* spp. which is morphologically very similar to *Engyodontium* spp. [4]. Our patient lived in a rural setting and reported considerable amount of outdoor activity; he was at increased risk of contracting a fungal infection from his environment. There is a remote possibility that he may have been infected at the time of his heart valve replacement, although modern methods of bioprosthetic valve chemical sterilization and microwave radiation have significantly reduced the incidence of *in situ* endocarditis [12–14].

Table 1 *In vitro* antifungal susceptibility data for case isolate.

Antifungal	MIC in µg/ml (72 h)	MIC in µg/ml (96 h)
Amphotericin B[1]	2	8
Anidulafungin[2]	1	1
Itraconazole[1]	0.5	>16
Ketoconazole[1]	0.5	2
Voriconazole[1]	0.5	2
Fluconazole[1]	>64	1

[1]Minimum inhibitory concentration (MIC).
[2]Minimum effective concentration (MEC).

Identifying the etiologic agent in fungal endocarditis is challenging as blood cultures are frequently negative [9] and often the diagnosis is only made *post mortem*. An extensive literature search for *Engyodontium album* and humans indicated that this is only the second reported case of *Engyodontium album* as a human pathogen [6] and the first case of bioprosthetic valve endocarditis. Genotypically, *Engyodontium album* is closely related to the insect pathogen *Beauveria bassiana* [10]. It is distinguished morphologically by its lack of discrete areas of conidiogensis seen with *Beauveria*, and by conidiogenous cells occurring in whorls. Obsolete names such as *Tritirachium album* and *Beauveria alba* attest to its morphological similarity to these genera. The difficulty in definitively identifying and separating *Engyodontium* from *Beauveria* was shown in this case as the isolate was initially misidentified as a *Beauveria* species. Furthermore, from a clinical perspective, it appears that both, *Engyodontium and Beauveria can* be isolated from sites with a core body temperature [6,10].

An additional challenge is determining appropriate therapy once the pathogen is isolated. Surgical intervention is usually required, and adjuvant antifungal chemotherapy for 6–8 weeks, or occasionally life-long therapy, is recommended [3,9]. AMB plus flucytosine (5-FC) remains the mainstay of therapy for *Candida* spp. causing endocarditis, but AMB does not penetrate well into vegetations [9] and there have been reports of viable fungal cells being cultured from resected valve tissue after 190 days of chemotherapy [9]. Voriconazole is the drug of choice for *Aspergillus* spp. endocarditis [3]. Recent evidence supports the use of echinocandins alone or in combination regimens for the treatment of *Candida* spp. endocarditis [2,15].

Although interpretive breakpoints regarding the MIC/MEC's do not exist for this organism, data based upon achievable serum concentrations using standard dosing regimens would suggest that the isolate was susceptible to ANID and possibly KTZ and VRZ. However, it was clearly resistant *in vitro* to FLZ and ITZ, as well as to the standard formulation of AMB. Liposomal preparations of AMB clearly achieve higher serum concentrations and could be efficacious. In our case, empiric antifungal therapy was based on the isolates initial identification and as such was directed against an *Acremonium* species. While the use of VRZ or AMB has been reported to be effective against members of this genus [16–18], it also appears to have been efficacious in our patient as there has been no evidence of relapse of his endocarditis. *In vitro* antifungal susceptibility data for our isolate was available only after the patient was nearing completion of his oral course of VRZ. We present a case of fungal endocarditis caused by an uncommon mould, *Engyodontium album*. There is no evidence of relapse following a combination of AMB, 5-FC, and VRZ antifungal therapy. It is also worth noting that echinocandins have not been extensively used for the treatment of mould infections [16,18–20]. However, based on the *in vitro* susceptibility data obtained for this isolate, anidulafungin demonstrated low MECs and may have also been an active agent against this filamentous organism.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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CASE REPORTS

Subcutaneous *Mycoleptodiscus indicus* Infection in an Immunosuppressed Dog▽

Catherine A. Metry,[1] Patricia S. Hoien-Dalen,[2] Carol W. Maddox,[3] Elizabeth H. Thompson,[4] Deanna A. Sutton,[4] Anna M. Romanelli,[5] Brian L. Wickes,[5] and Amy L. MacNeill[3]*

Department of Veterinary Clinical Medicine,[1] Veterinary Diagnostic Laboratory,[2] and Department of Pathobiology,[3] University of Illinois, Urbana, Illinois, and Fungus Testing Laboratory, Department of Pathology,[4] and Department of Microbiology,[5] University of Texas Health Science Center, San Antonio, Texas

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An 8-year-old dog presented with several dermal excoriations. Lesion cytology revealed pyogranulomatous inflammation with branching, septate hyphae. A mold identified as *Mycoleptodiscus indicus* by morphology and sequencing was cultured from fine-needle aspirates. This is the first report of a *Mycoleptodiscus* species as an etiologic agent in a dog.

CASE REPORT

An 8-year-old, outdoor, male, castrated pointer dog was presented to the University of Illinois Veterinary Teaching Hospital in April 2009 for recheck blood work 2 months after diagnosis of immune-mediated hemolytic anemia. Immunosuppressive treatment included daily oral administration of 2.1 mg prednisone/kg of body weight and 10.4 mg/kg cyclosporine (Atopica; Novartis Animal Health), along with 20 mg aspirin to prevent clot formation and 20 mg famotidine to limit gastrointestinal upset associated with the steroid administration. In addition, 200 mg doxycycline had been given twice daily for several weeks to treat for a possible underlying tick-borne illness.

The general physical exam revealed a potbellied appearance, hepatomegaly, and moderate to marked cachexia. The left rear leg was swollen, with pitting edema and a draining tract on the lateral aspect of the hock, the left popliteal lymph node was markedly enlarged, and several areas of minor dermal excoriations were present along the nasal planum. In addition, the dog's weight had decreased from 38.5 kg to 35 kg over the 2-month time period. Clinical differentials for the draining tract included phaeohyphomycosis, zygomycosis, pythiosis, lagenidiosis, sporotrichosis, and atypical bacterial infections, such as nocardiosis, actinomycosis, and mycobacteriosis.

Fine-needle aspirates were acquired from the left popliteal lymph node and from lesions on the bridge of the nose and the distal aspect of the left hind leg. Aspirates were submitted for cytologic evaluation, aerobic bacterial cultures, and fungal cultures. All cytology samples were Wright-Giemsa stained and were highly cellular, with a mixed population of severely degenerate neutrophils and reactive macrophages, including epithelioid macrophages and multinucleated giant cells. Bacterial cocci were noted extracellularly and within neutrophils. Aspirates from all sites contained basophilic, septate hyphae which measured 30 to 100 μm in length and 3 to 6 μm in width (Fig. 1). Some hyphae terminated in bulbous ends. The sample from the popliteal lymph node also contained several lymphocytes and rare, large, round yeastlike structures which rarely exhibited a narrow-base bud. The samples were interpreted as marked pyogranulomatous inflammation with bacterial and fungal sepsis.

Pending results of bacterial and fungal cultures, itraconazole was started at 5 mg/kg once daily, in addition to 32 mg/kg terbinafine once daily and 29 mg/kg cephalexin twice daily. The aerobic bacterial culture revealed a *Staphylococcus pseudintermedius* strain that was resistant to cephalexin but susceptible to clindamycin, amoxicillin-clavulanate, and enrofloxacin. Cephalexin was discontinued and clindamycin (12 mg/kg) once daily was started.

Fungal cultures at the University of Illinois Veterinary Diagnostic Laboratory recovered a rapidly growing mold on Sabouraud dextrose agar after 3 days of incubation at 25°C. The colonies had a uniform morphological appearance, consistent with isolation of a single species of fungus. By day 10, the colonies had a dark-brown reverse with a white woolly surface. Hyaline conidia were present at 2 weeks but were not readily identifiable, so internal transcribed spacer (ITS) sequencing was performed as described by Katsu et al. (6). A specific sequence match was not available in the NCBI GenBank database using the BLASTn algorithm in April 2009; therefore, the isolate was submitted to the Fungus Testing Laboratory at the University of Texas Health Science Center for additional sequencing and morphological studies under the culture collection number R-4334.

For morphological studies, the isolate was subcultured onto potato flake agar, prepared in-house, and incubated at 25°C. After 2 weeks, colonies were moderately fast growing, funiculous, and gray-yellow with patches of white and exhibited a

* Corresponding author. Mailing address: University of Illinois, Department of Pathobiology, 2001 S. Lincoln Ave., Urbana, IL 61802. Phone: (217) 244-3950. Fax: (217) 244-7421. E-mail: almac@illinois.edu.

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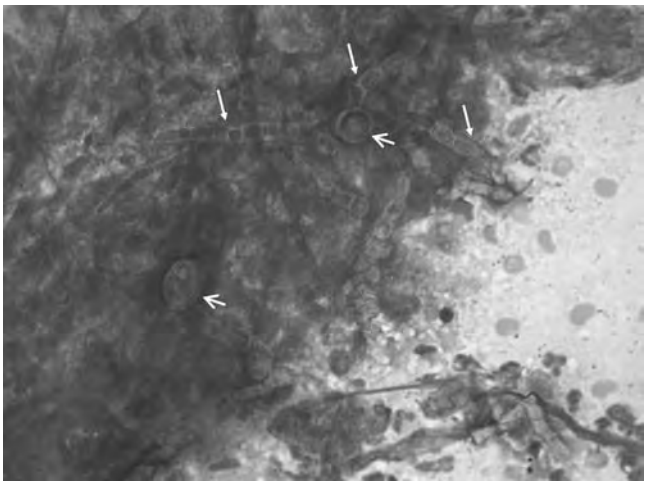

FIG. 1. Cytology of a fine-needle aspirate from a draining lesion on the left hind limb of a dog showing branching, septate hyphae (filled arrows). Bulbous structures at the terminal ends of the hyphae are also depicted (open arrows). Wright-Giemsa stain. Magnification, ×500.

yellow reverse and a diffusing yellow pigment. After 21 days, colonies were dark gray and the yellow pigment was no longer present. The isolate grew well at both 25°C and 35°C. Microscopically (on slide culture preparations), abundant appressoria and small aggregates of dark-brown cells (sporodochial conidiomata) were present. Appressoria were brown, smooth, not deeply lobed, and 0- or 1-septate (Fig. 2). Sporodochial aggregates were irregular in size and shape (Fig. 3A). Conidiogenous cells were broadly ovate or ampulliform with a prominent aperture and flared collarette (Fig. 3A and B). Conidia were hyaline, smooth, thin walled, broadly lunate, and mostly 0-septate (rarely 1-septate) (Fig. 3C) and measured 5 to 6 μm at the widest part and 16 μm to 20 μm long. The presence and number of polar and/or lateral appendages varied, ranging from none to polar at only one or both ends to polar and lateral on one or both sides (Fig. 3D). Appendage lengths varied and were up to 12 μm long. As the culture aged, conidial production began to decrease, with virtually no conidiogenesis occurring after week 4. The optimal time for observing conidia appeared to be within the first 1 to 2 weeks following subculture. Based upon the morphological features described above, the isolate was identified as a *Mycoleptodiscus* species (1, 10, 11).

Antifungal susceptibility testing was performed according to the method described in CLSI approved standard M38-A2 (3). Results read at 24 and 48 h were as follows: amphotericin B (0.125 and 0.25 μg/ml, respectively), itraconazole (0.25 and 0.5 μg/ml), voriconazole (0.25 and 0.5 μg/ml), posaconazole (<0.03 and 0.06 μg/ml), and terbinafine (0.015 and 0.015 μg/ml). Although no defined breakpoints are available for *Mycoleptodiscus* species, the isolate appears susceptible to all agents tested based upon normally achievable serum concentrations using standard dosing regimens.

Template DNA was prepared from a fresh subculture of R-4334, which had been grown on potato dextrose agar (Difco, Detroit, MI) for 20 h at 30°C. The sample was suspended in 50 μl of PrepMan Ultra reagent (Applied Biosystems, Foster City, CA) and processed according to the manufacturer's instructions. DNA amplification by PCR was performed in a total volume of 50 μl using 3 μl of the PrepMan supernatant as a template, high-fidelity Pfx50 DNA polymerase (Invitrogen, Carlsbad, CA), and primers ITS1 and ITS4, as previously described (12). In addition, D1/D2 amplicons were obtained using primers NL1 and NL4 and PCR conditions described previously (7, 9). PCRs were run in a PTC-100 thermocycler (MJ Research, Watertown, MA) using a three-step protocol, which consisted of 30 cycles with an annealing temperature of 58°C

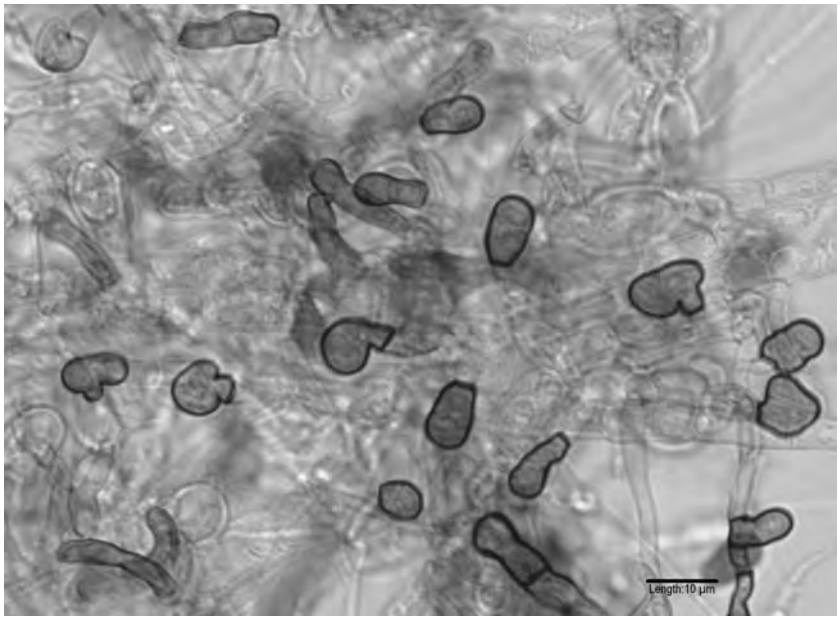

FIG. 2. Appressoria of this *Mycoleptodiscus* species as seen on a potato flake agar slide culture preparation mounted in lactophenol cotton blue.

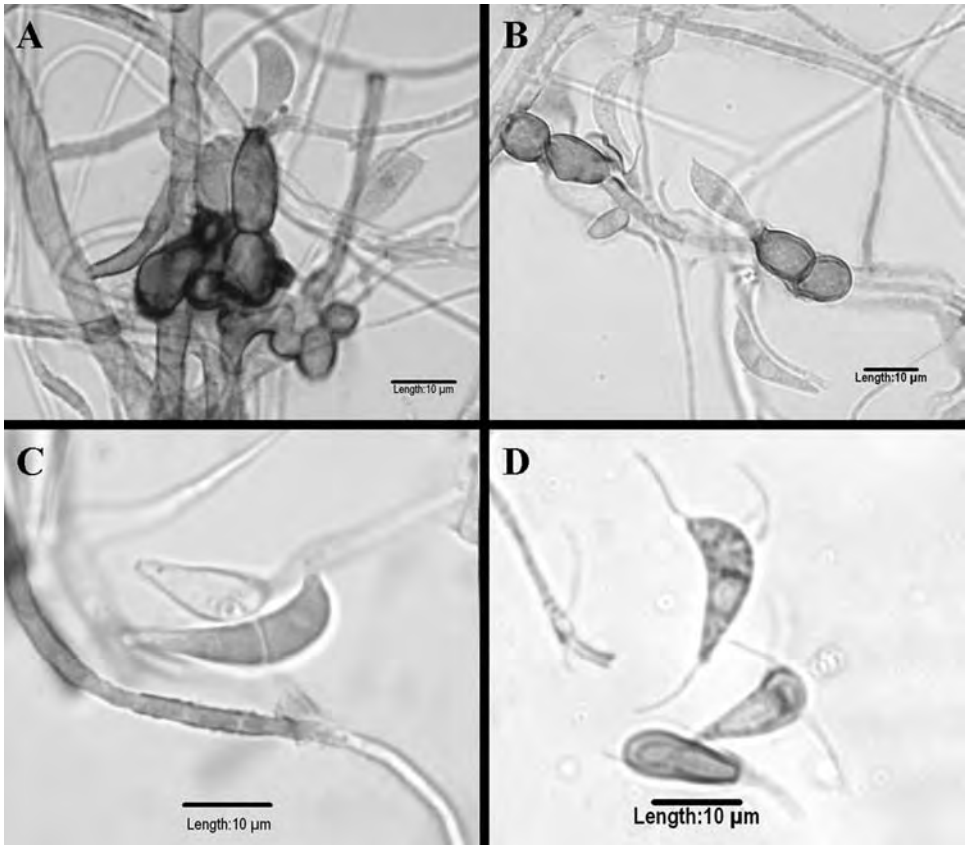

FIG. 3. Microscopic features of this *Mycoleptodiscus* species. (A) Small aggregate of sporodochial cells, a conidiogenous cell, and a conidium being released. Slide culture preparation mounted in lacto-fuchsin. (B) Conidiogenous cells with collarettes, and single-celled conidia with very short appendages. (C) Two-celled conidium. (D) Cellophane tape preparation demonstrating single-celled conidia with both polar and lateral appendages.

and a 1-min extension time. The PCR products were cleaned using a QIAquick PCR purification kit (Qiagen, Inc., Valencia, CA). Purified templates were sequenced in both directions at the University of Texas Health Science Center at San Antonio Advanced Nucleic Acids Core Facility using the same primers for ITS and D1/D2 amplification. Due to the paucity of sequence information available at the time of an initial search in May 2009, we were unable to confirm the morphological identification. However, a subsequent ITS search of the NCBI GenBank database in April 2010 revealed three hits at >99.5% identity (578/580, 576/577, and 567/568 nucleotides) at a >90% query coverage, corresponding to *Mycoleptodiscus indicus* (GenBank accession no. GU980698, GU980694, and GU980696). The next-closest species-level record was *Mycoleptodiscus terrestris*, which showed only 79% identity at a 100% query coverage. The D1/D2 BLASTn search showed similar results. Two records were found with 100% identity (616/616 nucleotides) at a query coverage of 100%. These two isolates were also *M. indicus* (accession no. GU980695 and GU980693). A third *M. indicus* record (accession no. GU980697) showed 99% identity but only at a 63% query coverage.

As for the outcome of this case, the dog was seen at the beginning of May 2009 and had multiple new subcutaneous and dermal nodules along the rib cage and distal limbs. Marked cellulitis was still present on the left distal rear limb. Abdominal ultrasound was performed and revealed hepatomegaly and a nonobstructive cholelith. Thoracic radiographs indicated that the dog had generalized megaesophagus. Radiographs of the distal aspect of the left tibia revealed cellulitis and a tibial malunion fracture with bony proliferation. The malunion fracture was thought to be an old injury; however, mild osteomyelitis could not be ruled out. Epsom salt soaks and whirlpool bath treatment were initiated in an effort to decrease the cellulitis, and mupirocin was started topically to help treat surface infections. At a subsequent visit, a slight increase in respiratory effort was noted and the dog's sclera had a mild jaundiced appearance, consistent with hemolytic anemia. The dog presented several days later to the emergency service for lethargy, regurgitation, and aspiration pneumonia secondary to megaesophagus. Clindamycin was discontinued, as it may have contributed to the development of generalized megaesophagus, and amoxicillin-clavulanate and enrofloxacin were prescribed to treat the aspiration pneumonia.

Due to the severe clinical signs of iatrogenic hyperadrenocorticism, several attempts were made to decrease the dosage of prednisone being administered to this patient, but with each attempt, the packed-cell volume decreased steadily over several days and there was evidence of hemolysis. As a result, the patient was maintained on oral prednisone (40 mg every 12 h)

and cyclosporine (400 mg daily), along with itraconazole, terbinafine, amoxicillin-clavulanate, and enrofloxacin, as previously described. The subcutaneous lesions waxed and waned during the course of treatment. The dog was discharged from the hospital on hospice care at the end of May and passed away at his home on 12 June 2009, approximately 2 months after diagnosis of this subcutaneous mycosis. A necropsy was not performed.

Anamorphic fungi in the genus *Mycoleptodiscus* are in the sexual-perithecium-forming family Magnaporthaceae. They are typically thought of as plant pathogens; however, *M. indicus* has been reported as an etiologic agent in immunosuppressed humans (5, 8) and, more recently, in a healthy human male (4). Infections are presumably acquired by traumatic implantation of the fungus.

Species identification of the etiologic agent in this case proved difficult. The most informative phenotypic features for identification of *M. indicus* include the presence of appressoria (Fig. 2) (also seen in a few other clinically significant molds, such as *Colletotrichum* species), dark phialidic, conidiogenous cells with prominent collarettes aggregated into sporodochial masses (Fig. 3A and B), and the production of large (10 to 16 by 5 to 7 μm), hyaline, typically single-celled, curved conidia (Fig. 3A and B). The production of septate conidia seen in the initial morphological workup suggested *M. terrestris* (10, 11) rather than *M. indicus.* The production of a yellow diffusible pigment on potato dextrose agar noticed in a subsequent examination in combination with the shape of the appressoria and the variable nature of the conidia also suggested two species with lateral appendages, namely, *Mycoleptodiscus variabilis* and/or *Mycoleptodiscus lateralis* (1).

Subsequent to submission of this report, ITS and D1/D2 nucleotide data for three human strains of *M. indicus* were deposited into GenBank. These three isolates showed the highest percent identities to our isolate, with all identities for both loci being >99.5%. These new deposits are from the University of Alberta Microfungus Collection and Herbarium. Since they are culture collection isolates and displayed good query coverage, there is high confidence in the sequence identification.

The identification of this isolate as *M. indicus* by sequence identity expands the known morphological features of this species, as conidia that are septate and/or exhibit lateral appendages have not been previously described. In addition, this is the first reported case of subcutaneous *M. indicus* infection in a dog.

Nucleotide sequence accession numbers. The D1/D2 sequence was deposited into GenBank under accession no. GU220383. The ITS sequence was deposited under accession no. GU220382. Our isolate was deposited into the University of Alberta Microfungus Collection and Herbarium under accession number UAMH 11157.

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Case Reports

Aureobasidium pullulans var. *melanigenum* fungemia in a pediatricpa tient

KILEENL . MERSHON-SHIER*, JAIMEG . DEVILLE†, SHIRLEY DELAIR†, ANNETTE W. FOTHERGILL‡, BRIAN WICKES§, G.SY BRENDE HOOG#, DEANNA A. SUTTON‡ & MICHAEL A. LEWINSKI*

**Department of Pathology & Laboratory Medicine & †Department of Pediatrics, University of California, Los Angeles, Los Angeles, California, ‡Fungus TestingL aboratory & §Microbiology and Immunology, University of Texas Health Sciences Center, San Antonio, Texas, USA, and #Centraalbureauvoor Sc himmelcultures,Ut recht, TheN etherlands*

This report describes a chronically ill child who presented with high fever and was diagnosed with catheter-related sepsis. *Aureobasidium pullulans* variety *melanigenum*, a dematiaceous fungus that rarely causes opportunistic infections, was recovered from multiple blood cultures. Antifungal susceptibilities were performed and the minimum inhibitory concentration (MIC) for fluconazole was 64 mg/l, suggestive of fluconazole resistance. The patient made a full recovery after removal of the catheter line and treatment with liposomal amphotericin B. This is the first case report of an elevated *in vitro* fluconazole MIC of an *A. pullulans* isolate and only the third case of successful treatment of *A. pullulans*f ungemia.

Keywords *Aureobasidium*, fungemia, fluconazole

Introduction

Aureobasidium pullulans is a dematiaceous fungus that is found in plant debris, soil, wood, textiles, and indoor air. It is often considered a contaminant in microbiology cultures, but can also be a source of infection in immunocompromised hosts. Reported clinical manifestations include keratomycosis, cutaneous mycoses, peritonitis, meningitis, and fungemia [1]. Thirteen cases of systemic infection have been described and all but one of the patients had a documented intravascular device [1,2], suggesting that catheters play a role in *A. pullulans* infections.

This report describes a chronically ill child who presented with signs and symptoms of intravascular catheter-related bloodstream infection. *A. pullulans* variety *melanigenum* was recovered from multiple blood cultures, which is consistent with previous reports of catheter-associated *Aureobasidium* infections. However, this is the first report of an *A. pullulans* isolate with an elevated *in vitro* susceptibility to fluconazole MIC of 64 mg/l, suggesting some strains of *A. pullulans* may be resistant to fluconazole.

Case report

An 11-year-old boy with a history of intestinal lymphangiectasia, protein losing enteropathy and lymphopenia presented with a 1-day history of fever of up to 40.1°C and chills. Due to his total parenteral nutrition-dependency, he had a Hickman catheter which had been originally placed 7 months prior to his admission. His catheter site was without erythema, warmth or discharge. He was admitted with suspected intravascular catheter-related bloodstream infection and started empirically on vancomycin, piperacillin-tazobactam, and gentamicin.

After 30 h of incubation, one aerobic BacT/Alert bottle (bioMérieux, Marcy l'Etoile, France) and one fungal BACTEC bottle (BD, Franklin Lakes, New Jersey) inoculated with samples from his Hickman catheter yielded yeast-like organisms which were visualized by wet mount and Calcofluor (Polysciences, Inc., Warrington, Pennsylvania) staining. The same organism was recovered after 11 days of incubation from a fungal BACTEC blood culture bottle

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Correspondence: Kileen L. Mershon-Shier, University of California, Los Angeles, Department of Pathology & Laboratory Medicine, Los Angeles, CA, USA. E-mail: kmershon@mednet.ucla.edu

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inoculated 19 h after the first set. The isolate was subcultured to Sabouraud brain heart infusion agar (BD catalog #298192) and inhibitory mold agar (BD catalog #298191) and incubated at 30°C overnight. The next day, colonies were found on both media and the fungus was found to be germ tube test negative. As a result, the isolate was subcultured to a cornmeal Tween 80 agar (manufactured in-house) and incubated at 30°C for 48 h. The colonies that grew were yeast-like in consistency and peach in color, but developed a black color as the colony aged (Fig. 1). On cornmeal Tween 80 agar, there were tiny hyaline blastoconidia and dark septated hyphae, suggestive of the genera *Hormonema* or *Aureobasidium* (Fig. 2). The isolate was reported as a *Hormonema* species due to a negative methyl-α-D-glucoside assimilation test in an API 20C AUX panel (bioMérieux) which had been incubated at 30°C for 72 h. The results are consistent with *Hormonema dematioides* [3].

An isolate of the yeast-like organism was referred, 7 days after the specimen was collected from the patient, to the Fungal Testing Laboratory of the University of Texas Health Science Center – San Antonio for antifungal susceptibility testing, and accessioned into their culture collection as UTHSC 09-1190. Testing was accomplished according to methods outlined in CLSI document M38-A [4] modified to a macrobroth dilution method. The results (Table 1) were available 10 days after the isolate was submitted, a total of 17 days after it was collected. Thus the results were not available to the physicians while the patient was hospitalized.

The patient was started on liposomal amphotericin B at 4 mg/kg once daily when the first set of blood cultures were

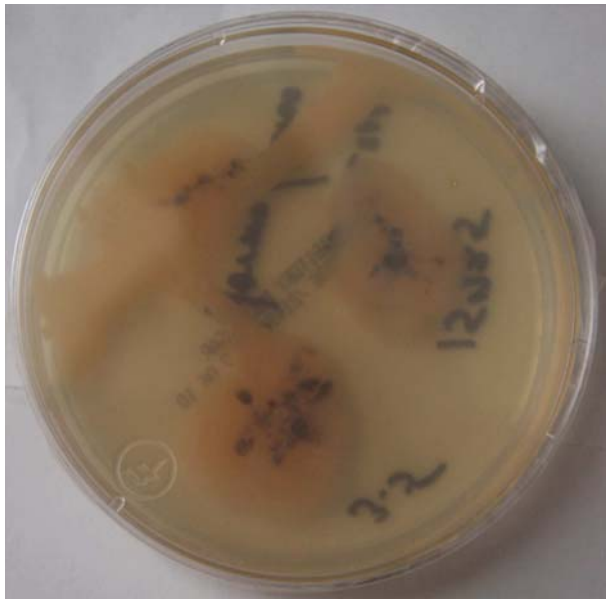

Fig. 1 Macroscopic morphology of *Aureobasidium pullulans* var. *melanogenum* on inhibitory mold agar.

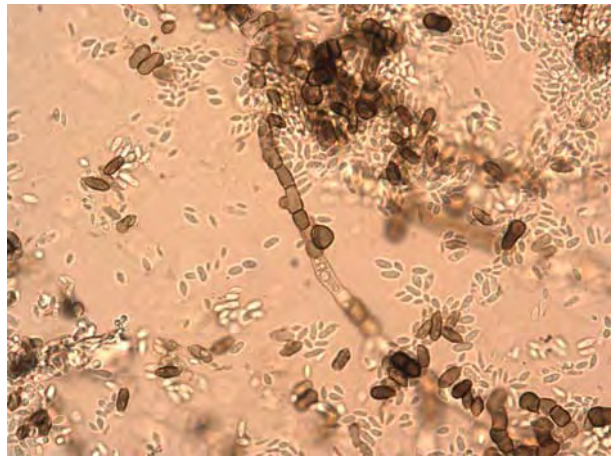

Fig. 2 A tease prep of *Aureobasidium pullulans* var. *melanogenum* at ×400 showing dark septated hyphae and small hyaline blastoconidia.

reported to contain fungal elements. Amphotericin B treatment was continued after identification of the isolate based on the patient's clinical improvement and reports of the *in vitro* resistance of the isolate to fluconazole [5,6]. A fungal blood culture drawn after amphotericin B treatment was negative. The patient defervesced and his catheter was removed the following day, with no microorganisms recoverable in bacterial and fungal cultures from the catheter tip. Two sets of peripheral blood cultures, one bacterial and one fungal, were sterile after the removal of the catheter and there was no evidence of dissemination. The patient received 2 weeks of amphotericin B therapy and was discharged uneventfully.

To confirm the identification of the organism, DNA sequencing of the internal transcribed spacer (ITS) and D1/D2 regions was performed with an isolate under the accession number R-4360. A comparison of the sequencing data with the Centraalbureau voor Schimmelcultures (CBS) sequence database in July 2009 identified the isolate as having 99.8% ITS similarity with CBS 105.22, *A. pullulans* var. *melanogenum* [7].

Discussion

Colonies of the dematiacous fungus *A. pullulans* are initially white to pink, with a creamy yeast-like consistency.

Table 1 Results of the antifungal susceptibility testing.

Drug	MIC (mg/l) at 48 h
Amphotericin B	0.25
Caspofungin	4
Fluconazole	64
Posaconazole	0.125
Voriconazole	0.5

At maturity, areas of brown to black develop and are seen in the colonies. Microscopic morphology is initially unicellular, budding, and yeast-like in appearance. However, yeast-like cells develop into hyaline, delicate, and thin-walled vegetative hyphae, which produce blastoconidia, or dark, thick-walled, closely septate intercalary segments or discrete chlamydospores. *A. pullulans* closely resembles *H. dematioides* in both colonial and microscopic morphology. However, *A. pullulans* conidia form mostly on hyaline hyphae whereas *H. dematioides* conidia form on both hyaline and dematiaceous hyphae. Additionally, *A. pullulans* conidia develop synchronously from separate fertile points while those of *H. dematioides* develop asynchronously from a single fertile point [3]. However, these microscopic distinctions are subtle and difficult to demonstrate. Assimilation of methyl-α-D-glucoside (MDG) is also thought to be a reliable test to differentiate the two organisms, with *A. pullulans* being positive and *H. dematioides* being negative [3]. However, our isolate was MDG negative, suggesting *H. dematioides*, but unambiguously identified as *A. pullulans* by DNA sequencing, demonstrating a discrepancy between its biochemical property and sequenced-based identification.

A. pullulans has been reported to cause a variety of infections including peritonitis, meningitis, and fungemia [1]. Of the 32 cases of human infections reported in the English literature, 13 were systemic with positive blood cultures [1,8–12] and only two of those involved pediatric patients [1,11]. All but one of the patients had intravascular devices, suggesting that such devices are a risk factor for *A. pullulans* fungemia [1,2,8–12]. Previous studies have shown that *A. pullulans* is capable of forming biofilms which can adhere to catheters [13], suggesting a role for intravascular devices in *A. pullulans* fungemia. The positive blood cultures from our patient were drawn through a Hickman catheter, consistent with the previous findings of catheter-associated fungemia.

Interestingly, the isolate from our patient had an *in vitro* fluconazole MIC of 64 mg/l. Only one previous case reported antifungal susceptibilities for an isolate from a systemic *A. pullulans* infection and it had a four-dilution lower fluconazole MIC of 4 mg/l [1]. While there are no established interpretive criteria for antifungal susceptibilities for *A. pullulans*, the high MIC of our isolate suggests fluconazole resistance. This is consistent with reports of the related fungus *H. dematioides* being unresponsive to fluconazole therapy [5,6]. It is important to note that these isolates were identified morphologically prior to the availability of molecular sequencing confirmation, thus it is possible some of the previously identified *H. dematioides* isolates were actually *A. pullulans*. This case report demonstrates the need to use empiric fluconazole therapy with caution, as some fungi are fluconazole-resistant.

Voriconazole has also been used to successfully treat classic phaeohyphomycosis, though not specifically *Aureobasidium* infections [14]. In the only report of *A. pullulans* fungemia treated with voriconazole, the patient continued to have fevers after 6 days of treatment and was switched to liposomal amphotericin B with resolution of fever after an additional 6 days [11]. This suggests voriconazole may not be the optimal therapy, although no susceptibilities were performed on this isolate. The MIC for voriconazole of our patient's isolate was 0.5 mg/l, suggesting susceptibility, though no breakpoints are established.

Amphotericin B has been used successfully to treat *A. pullulans* infections involving one presumed systemic dissemination, two of meningitis, two of peritonitis [1], and two proven cases of fungemia in which the lines were removed [10,11]. Amphotericin B treatment was unsuccessful in three additional cases of fungemia in which the lines were not removed [1,9,12]. The previously reported *in vitro* antifungal sensitivities of this fungus indicated an amphotericin B MIC of 0.5 mg/l [1]. In the latter case, the patient was treated with amphotericin B for 40 days without line removal, but died 13 days later due to chronic lung disease. On autopsy, intravascular fungal hyphae resembling *A. pullulans* were seen, indicating the patient was not able to clear the infection [1]. Our patient's isolate had an amphotericin B MIC of 0.25 mg/l and he was able to clear the infection through the use of liposomal amphotericin B therapy and removal of his line.

Treatment of one case involving the use of fluconazole and the line removal was also successful [8]. This suggests that the removal of the intravascular catheter plays a critical role in resolution of the infection. However, in our patient, blood cultures for fungi became negative after amphotericin B treatment even prior to line removal. The line was still removed due to concern for recurring infection. It is possible that amphotericin B can decrease the level of fungemia below detection, but cannot completely eliminate the infection, possibly due to the formation of a biofilm on the catheter. This would render a patient with a remaining catheter line susceptible to recurring infection.

This is the first documented case of *Aureobasidium* fungemia in which the etiologic agent had an elevated *in vitro* fluconazole MIC and only the third case of *A. pullulans* fungemia to respond to amphotericin B treatment in combination with line removal. Additionally, this is the first isolate of *A. pullulans* identified to variety level by DNA sequencing. Isolate UTHSC 09-1190 has been deposited into the CBS Fungal Biodiversity Center under the accession number CBS 125735. Both the ITS and D1/D2 sequences were submitted to Genbank under accession numbers GU475133 and GU475134, respectively.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Disseminated Human Conidiobolomycosis Due to *Conidiobolus lamprauges*[▽]

Masatomo Kimura,[1]* Takashi Yaguchi,[2] Deanna A. Sutton,[3] Annette W. Fothergill,[3] Elizabeth H. Thompson,[3] and Brian L. Wickes[4]

Department of Pathology, Kinki University Faculty of Medicine, Osaka-Sayama, Japan[1]; Medical Mycology Research Center, Chiba University, Chiba, Japan[2]; and Fungus Testing Laboratory, Department of Pathology,[3] and Department of Microbiology and Immunology,[4] University of Texas Health Science Center at San Antonio, San Antonio, Texas

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We describe a disseminated fungal infection by *Conidiobolus lamprauges* in a patient with malignant lymphoma. Histopathology and mycological studies were performed, along with molecular analyses. This is the first record of this species causing human disease and the fifth reported disseminated infection by a *Conidiobolus* sp. in humans.

CASE REPORT

A 61-year-old male Japanese office worker was diagnosed with relapsed mantle cell lymphoma with bone marrow infiltration. Since repeated chemotherapy did not achieve complete remission, the patient finally received a nonmyeloablative allogeneic unrelated hematopoietic stem cell transplant. Short-term methotrexate treatment was employed for graft-versus-host disease prophylaxis. On day 7 posttransplant (PT), severe ($<0.1 \times 10^3$ neutrophils/μl) neutropenia was noted. Treatment with broad-spectrum antibiotics and micafungin (150 mg/day) was initiated for febrile neutropenia and continued until day 20 PT. Neutrophil engraftment occurred on day 16 PT. Cytomegalovirus antigenemia was detected and treated with ganciclovir from day 39 until day 48 PT. On day 47 PT, pancreatitis developed and was treated with anticoagulant therapy and anti-pancreatic-enzyme therapy for about a week. Treatment with broad-spectrum antibiotics was started on the same day and continued, using various antibiotics, until 4 days before the patient died. A chest X-ray film demonstrated bilateral lung infiltration on day 53 PT, with elevation of serum (1→3)-β-D-glucan (BG) levels to 27.0 pg/ml (as determined by the β-D-glucan Wako test) (Wako Pure Chemical Industries, Tokyo, Japan) (normal levels ≤ 10 pg/ml). Since fungal infection was suspected, treatment with micafungin (150 mg/day) was restarted on day 53 PT. One week later, this was replaced by treatment with liposomal amphotericin B (2.5 mg/kg/day) because a blood culture yielded *Candida albicans* and because the serum BG levels had increased to 63.6 pg/ml. On day 62 PT, treatment with hydrocortisone was initiated for hemophagocytic syndrome therapy. Treatment with pentamidine isethionate was started on day 70 PT and continued until the death of the patient, likely due to suspected *Pneumocystis* pneumonia. On day 74 PT, antifungal therapy was switched from liposomal amphotericin B to a combination of voriconazole (loading dose, 6 mg/kg, followed by 4 mg/kg [administered intravenously every 12 h]) and micafungin (150 mg/day) because the serum BG levels had increased significantly to 2,366.0 pg/ml and a chest X-ray film demonstrated additional widespread pulmonary infiltration. Severe ($<0.1 \times 10^3$ neutrophils/μl) neutropenia due to hemophagocytic syndrome was recorded from day 76 PT. The patient's general condition worsened, and the patient died of respiratory failure on day 80 PT.

An autopsy was performed 2 h and 36 min after death. Pathological examination demonstrated that the mucosa of the trachea and the main bronchi was eroded. All lobes of the bilateral lungs were congested with irregular multiple hemorrhages and 5- to 10-mm-diameter nodular infarcts. Infarcts were also found in the heart, bilateral kidneys, spleen, and thyroid gland. A filamentous fungus (*Conidiobolus lamprauges*) was isolated from the tissues of the tracheal mucosa and the infarcted lesions presenting in the bilateral lungs, bilateral kidneys, and spleen. *Enterococcus faecium* and *Staphylococcus aureus* were cultured from the mucosa of the trachea, bilateral lungs, and bilateral kidneys. *E. faecium* was also cultured from the spleen and blood.

In microscopic analyses, fungal hyphae were found to be proliferating in the tracheobronchial tissue and the lungs, heart, kidneys, urinary bladder, urethra, spleen, and thyroid gland, where they were markedly invading blood vessels. Consequently, mycotic thrombi were frequently found, causing nodular infarcts (Fig. 1A). There was no apparent inflammatory cell infiltration in the lesions associated with fungal infection. There were two different types of hyphal morphology in the tissue. One type was in the form of widely distributed Mucorales-like broad (4- to 13-μm-wide) thin-walled pauciseptate hyphae with irregular branching (Fig. 1B). These were often wrinkled, folded, and twisted, and the tips of the hyphae sometimes showed unusual bulbous dilation (21 to 40 μm in diameter) (Fig. 1C). The other type was represented by dichotomously branching 5- to 7-μm-wide septate hyphae with parallel sides resembling those of *Aspergillus* morphology; these were mostly seen in the mycotic thrombi (Fig. 1D). None of the hyphae in the tissue were coated by a deposit of eosinophilic Splendore-Hoeppli material. The liver, gallbladder, alimentary

* Corresponding author. Mailing address: Department of Pathology, Kinki University Faculty of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan. Phone: 81-72-366-0221, ext. 3142, 3141. Fax: 81-72-360-2028. E-mail: kimura-m@med.kindai.ac.jp.

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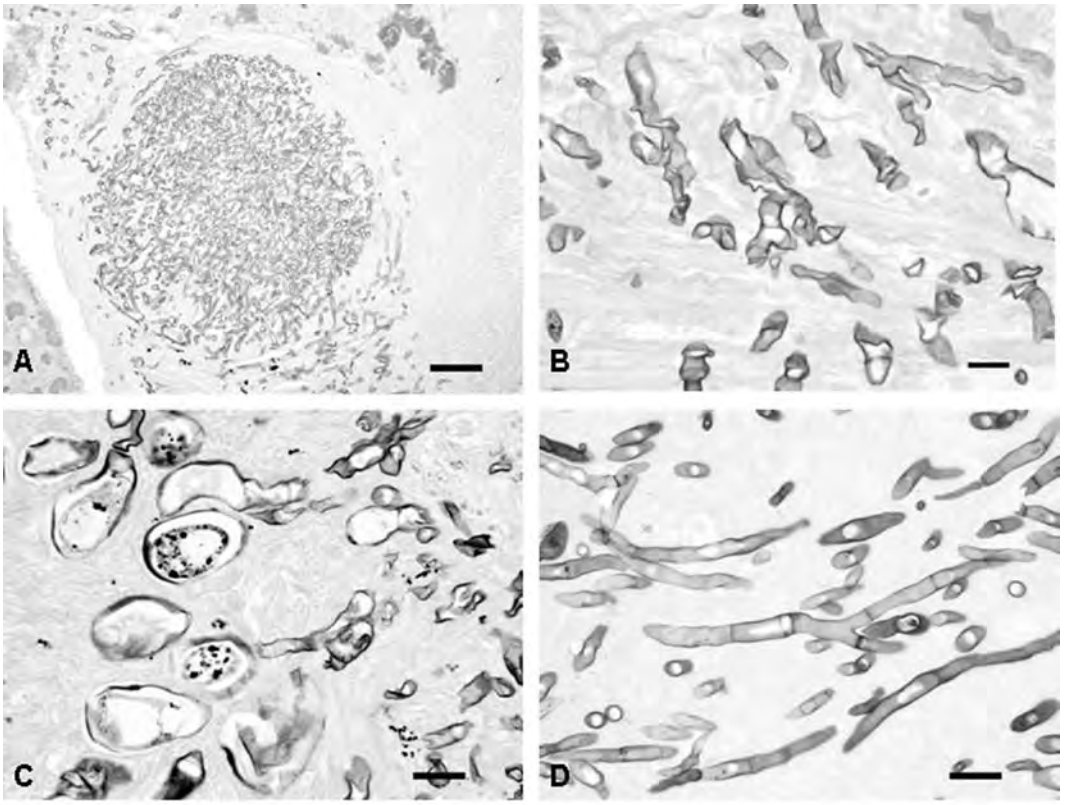

FIG. 1. Histopathology of *Conidiobolus lamprauges* invading pulmonary tissue. (A) The vascular lumen is occluded by a mycotic thrombus composed of numerous hyphae. (Grocott staining; bar, 100 μm.) (B) Hyphae showing irregular branching with frequent wavy shapes characteristic of Mucorales hyphae. (Grocott staining; bar, 20 μm). (C) The tips of many hyphae show unusual bulbous dilation. Nondilated hyphal portions are continuous with the dilated portions. (Grocott staining; bar, 25 μm). (D) Proliferating hyphae, resembling *Aspergillus* hyphae, in a vascular lumen. The hyphae are septate, have parallel sides, and branch dichotomously at acute angles. (Grocott staining; bar, 25 μm).

tract, and genital organs were spared fungal infection. No lymphoma cells were detected in any of the tissues examined.

An autopsy isolate recovered from the pulmonary lesions was forwarded to the Fungus Testing Laboratory at the University of Texas Health Science Center at San Antonio (UTHSCSA) (TX) for morphological and molecular characterization and was added to their culture collection under accession number UTHSC R-4463. Morphological and temperatures studies were conducted on potato flakes agar (PFA) prepared in-house. Colony diameters were measured on 100-mm-diameter petri dishes incubated at 24, 36.5, 40, 45, and 50°C. Growth was rapid, and colonies were pale, thin, effuse, and glabrous within the first 72 h (Fig. 2A), with average colony diameters at 24, 48, and 72 h as follows: at

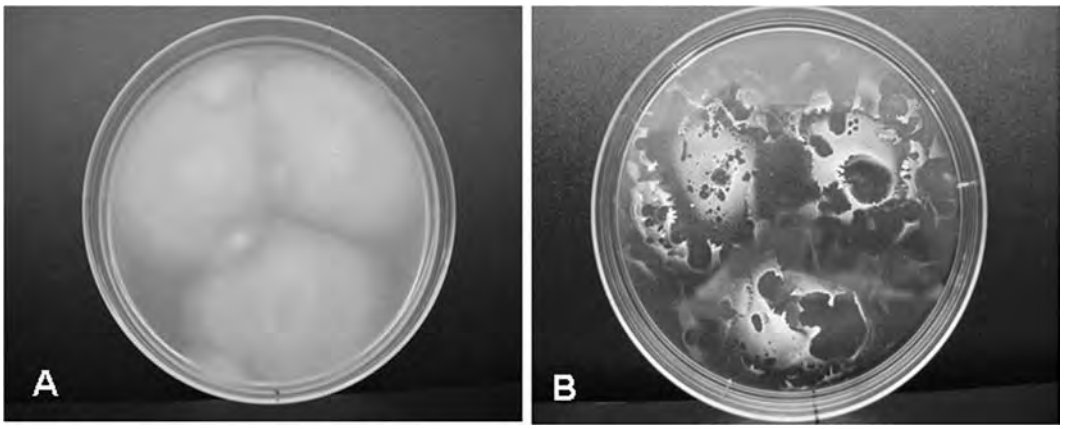

FIG. 2. Colonial morphology of *Conidiobolus lamprauges* on PFA after 72 h of incubation at 36.5°C. (A) The colony is pale, thin, effuse, and glabrous. (B) Lid of petri dish, demonstrating forcibly discharged conidia of *Conidiobolus lamprauges*.

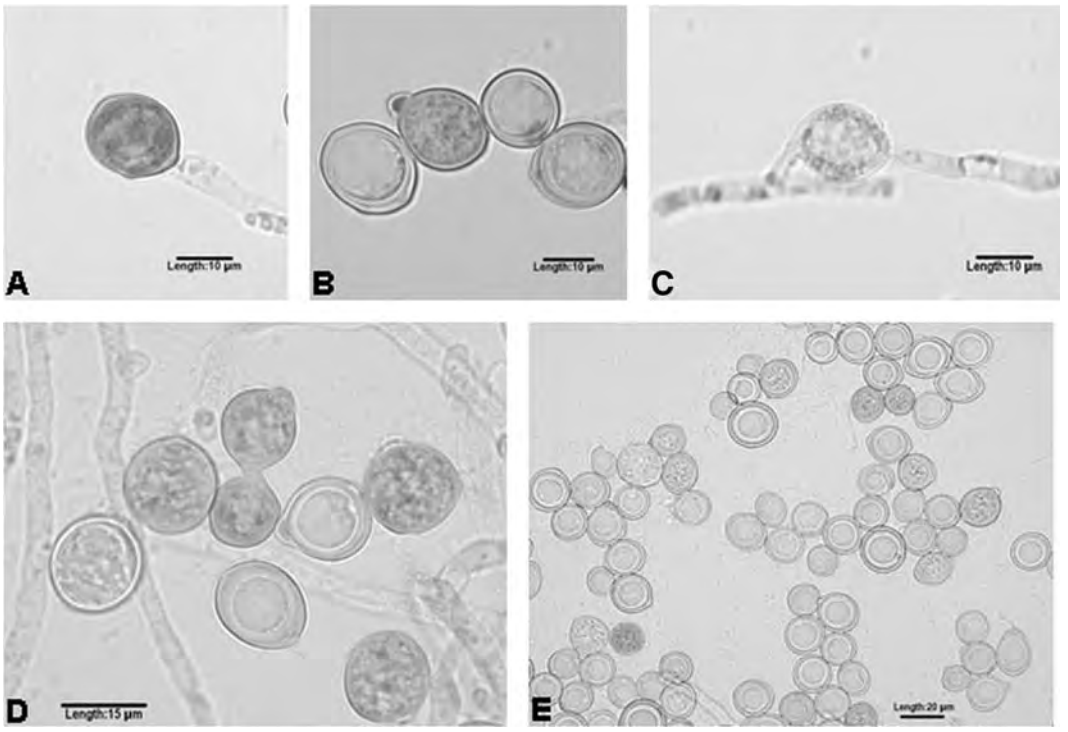

FIG. 3. Microscopic morphology of *Conidiobolus lamprauges*. (A) Conidiophore and primary conidium prior to release. (B) Zygospores containing a large globule and a single papillate primary conidium. (C) Immature zygospore formed between two hyphal segments. (D) Hyphae, zygospores (with large globules), and primary conidia, one of which shows migration of the cytoplasm from the primary conidium into the secondary replicative conidium. (E) Zygospores and conidia at lower magnification.

24°C, 10 mm, 25 mm, and 33 mm, respectively; at 36.5°C, 23 mm, 40 mm, and 52 mm, respectively; and at 40°C, 20 mm, 32 mm, and 40 mm, respectively. The lid of the petri dish demonstrated forcibly discharged conidia (Fig. 2B). No growth occurred at 45 or 50°C after 72 h. The isolate also failed to grow on media containing 10 μg/ml benomyl, prepared in-house, but did exhibit growth on media containing 0.04% cycloheximide (Remel, Lenexa, KS). Colonies became pale yellowish-beige with extended incubation. Microscopic features observed on a PFA slide culture preparation and tease mounts in lactophenol cotton blue are shown in Fig. 3 and included conidiophores (4.8 to 7.2 μm in width) and primary conidia prior to release (Fig. 3A); zygospores (14 to 29 μm, with an average diameter of 24 μm) containing a large globule and a single papillate primary conidium (15 μm in diameter) (Fig. 3B); an immature zygospore formed between two hyphal segments (Fig. 3C); hyphae, zygospores (with large globules), and primary conidia, one of which showed migration of the cytoplasm from the primary conidium into the secondary replicative conidium (Fig. 3D); and zygospores and conidia visible at lower magnification (Fig. 3E). Villose conidia and multireplicative conidia were absent. Our isolate was microscopically identical to those reported by others using the features proposed by Vilela et al. (14). On the basis of the macroscopic, microscopic, and physiologic features cited above, the isolate was morphologically identified as *C. lamprauges*. The isolate has been deposited into the University of Alberta Microfungus Collection and Herbarium under accession number UAMH 11219 and the Medical Mycology Research Center, Chiba University, Japan, under accession number IFM58391.

Isolates were prepared for sequence analysis first by subculture onto potato dextrose agar (PDA) plates and then by incubation for 24 h at 30°C. Template DNA was isolated from PDA plates as described previously (12). PCR was performed using template DNA and primers ITS1 and NL4 to amplify a single contig containing the internal transcribed spacer (ITS) and D1-D2 regions, and the amplified material was then sequenced on both strands with primers ITS1, ITS4, NL1, and NL4 at the UTHSCSA Advanced Nucleic Acids Core Facility (12). The ITS and D1-D2 sequences were then used to search the GenBank database at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLASTn algorithm. Results were sorted according to the percentage of maximum identity and were considered significant for query coverage of >90% and maximum identity of >97%. The top four hits from the ITS BLASTn search were all for *C. lamprauges* (accession numbers GQ478279, GQ478280, GQ478281, and AF296754) at 100%, 100%, 99%, and 99% identities, respectively, with hits ranging from 749 of 750 bp to 750 of 750 bp. The next closest ITS hit was *Schizangiella serpentis* at 90% identity but only 79% query coverage. The D1-D2 search returned nine *Conidiobolus* hits, with the top hit being *C. lamprauges* at 100% identity and 100% query coverage (accession number AF113458), with 643 of 643 bp matching. The remaining *Conidiobolus* species (*C. thromboides*, *C. osmodes*, *C. antarcticus*, and *C. pumilus*) all displayed identities of <84% and query coverage of 80% or less and were therefore not

considered significant. Based on the BLASTn search, the identity of our isolate, UTHSC R-4463, was consistent with *C. lamprauges*. (Sequence data have been deposited in the GenBank database; see below).

Antifungal susceptibility testing was performed according to the guidelines outlined in the CLSI M38-A2 document (2). The concentration of the inoculum was analyzed using a spectrophotometer and then adjusted to a final value of 1 to 5×10^4 CFU/ml in RPMI 1640 medium. The inoculum was then added to round-bottomed microtiter wells containing various concentrations of antifungal agents, including amphotericin B (AMB), anidulafungin (ANID), caspofungin (CAS), micafungin (MICA), itraconazole (ITC), voriconazole (VRC), posaconazole (POSA), miconazole (MON), and terbinafine (TRB). Plates were incubated at 35°C for 48 h, with endpoint readings determined at both the 24- and 48-h time points. The endpoint was determined as the lowest concentration that completely inhibited growth (AMB, ITC, VRC, and POSA), that resulted in a 50% inhibition of growth (MON and TRB), or that resulted in aberrant growth (ANID, CAS, and MICA) (i.e., the minimum effective concentration [MEC]). Combination testing was accomplished for ITC plus TRB and for POSA plus TRB. Parameters outlined in the M38-A2 document were used in the checkerboard dilution format. Since different endpoints were assessed for TRB versus the azoles when individual drug activity was assessed, the more stringent 100% inhibition endpoint value was used in determining endpoints for combination studies. The results obtained at 24 and 48 h, respectively, were as follows: AMB, 4 and 8 μg/ml; ANID, >8 μg/ml; CAS, >8 μg/ml; MICA, >8 μg/ml; ITC, 0.5 and 1 μg/ml; POSA, 4 and 4 μg/ml; VRC, >16 μg/ml; MON, 0.25 and 1 μg/ml; and TRB, 0.03 and 0.125 μg/ml. Although no breakpoints have been defined for this organism, interpretive guidelines based upon achievable drug concentrations suggest resistance to all agents *in vitro*, possibly excluding ITC, MON, and TRB. Results of synergy studies with ITC and TRB at 1 and 0.06 μg/ml and POSA and TRB at <0.03 and 0.125 μg/ml were interpreted as indifferent (i.e., neither synergistic nor antagonistic).

Discussion. Conidiobolomycosis is an infectious disease caused by a fungus belonging to the genus *Conidiobolus* within the order Entomophthorales and in the class Zygomycetes (7). Members of the genus *Conidiobolus* are generally considered saprobes distributed in plant detritus and soil (7, 11, 13). Three species in the genus *Conidiobolus* are known to cause diseases in humans or animals: *C. coronatus*, *C. incongruus*, and *C. lamprauges* (5, 7, 14). This report is the first record of *C. lamprauges* causing human disease and the fifth reported disseminated infection caused by a *Conidiobolus* sp. in humans. Human infection with *Conidiobolus* species occurs most commonly as chronic rhinofacial mycosis in otherwise healthy hosts (7, 11). Disseminated human infections have previously been described in only four cases, with the etiological agent being *C. incongruus* in two cases, *C. coronatus* in one case, and an unidentified *Conidiobolus* sp. in the other case (1, 6, 15, 16). *C. lamprauges* has previously been implicated as the causative agent of nasopharyngeal infection in horses and sheep (5, 14). The modes of infection by *C. lamprauges* were quite different between these animal cases and the present human case. These differences may be related to factors such as the host immune status, human susceptibility to certain strains, and the ability of these strains to adhere to human tissues. The patient described in this case report was immunocompromised, and that may have been a predisposing factor in disseminated infection. In addition, the ability of our strain to invade blood vessels might have been superior to that of animal strains.

The mode of transmission of the *Conidiobolus* species was not established in the present case. Since *C. lamprauges* is usually found in leaf litter and soil (13), inhalation of its airborne conidia could have been the route of transmission in this case. This possibility was supported by the autopsy finding of tracheobronchial erosion with fungal invasion and bilateral fungal pneumonia. Given that the patient had been hospitalized for approximately 50 days before the detection of pulmonary infiltrate on a chest X-ray film and the elevation of the serum BG level, this infection may have been acquired in the hospital. However, there was no apparent event, such as ongoing hospital construction, that might have resulted in pollution of the room air with fungal elements.

An important laboratory finding in the present case was the detection of serum BG, and its increased level, along with the burden of infection. BG is a cell wall polysaccharide component of most fungi and can be detected in the bloodstream of patients with fungal infections (8). It can be a surrogate marker of invasive fungal infections, and its monitoring helps to assess the effectiveness of antifungal therapy (10). Since the zygomycetes and *Cryptococcus* species have lower BG content, BG detection assays are often less useful (9). Even though *Conidiobolus* species are members of the zygomycetes, the serum BG in this patient increased to an extremely high level. To date, there have been no other reports regarding serum BG levels during conidiobolomycosis, and more cases need to be accumulated before serum BG levels during conidiobolomycosis can be thoroughly assessed.

The most interesting histological point raised by the present case was that the fungal hyphae of the *Conidiobolus* species masqueraded as both Mucorales and *Aspergillus* species in tissue. Widely distributed hyphae were thin-walled, broad, and pauciseptate, with irregular branching, resembling the hyphae of species of Mucorales. The same features have been described in reports of three previous immunocompromised patients with disseminated conidiobolomycosis (6, 15, 16). Due to the shape of the hyphae and the lack of Splendore-Hoeppli material surrounding them, *Conidiobolus* hyphae are indistinguishable from those of the Mucorales species in tissue. In contrast, many of the hyphae proliferating in the vascular lumens were uniform and septate with dichotomous branching (Fig. 1D), resembling those of *Aspergillus* species. Thus, histologic features must be interpreted with caution.

Although Vilela et al. (14) showed colonies with "radial folds," no such colony morphology was observed in our case. Colonial morphology is often dependent on the media used, and the photomicrographs by Vilela et al. (14) were taken on Sabouraud dextrose agar versus the PFA used in our case, possibly explaining these differences. It should also be noted that the radial folds they describe are more evident with ex-

tended incubation (i.e., 6 days), whereas our images were recorded at 3 days. A key feature in the presumptive morphological identification of both *Conidiobolus* and *Basidiobolus* spp. is the detection of forcibly discharged conidia on the lid of the petri dish (Fig. 2B). The finding of a single, large globule within mature zygospores (Fig. 3B, D, and E), a feature not present within those of either *C. incongruus* or *C. coronatus*, serves as a presumptive identification of *C. lamprauges*. Our isolate was microscopically identical to those reported by others using the features proposed by Vilela et al. (14).

There is no consensus regarding the appropriate antifungal treatment for *Conidiobolus* infection. Cotrimoxazole, AMB, and AMB with flucytosine have each been used against disseminated infection but with no success (1, 15, 16). The present fungal infection did not respond to therapy that should have been effective against Mucorales and *Aspergillus* species. Our *in vitro* antifungal susceptibility studies suggested that our isolate was multidrug resistant, explaining the therapy failure. The *in vitro* antifungal susceptibilities of seven isolates belonging to *Conidiobolus* spp., including one isolate of *C. lamprauges*, to six antifungals (AMB, ketoconazole, MON, ITC, fluconazolem and flucytosine) were tested by Guarro et al., and all of the isolates were resistant to all of the antifungals (4). The results of our synergy studies performed with ITC plus TRB at 1 plus 0.06 μg/ml and POSA plus TRB at <0.03 plus 0.125 μg/ml were interpreted as indifferent (i.e., neither synergistic nor antagonistic). According to a recent clinical report, treatment with a combination of ITC and TRB resulted in the successful treatment of rhinofacial *C. coronatus* infection, although *in vitro* susceptibility testing of the isolate revealed resistance to both ITC and TRB (3). Since such combination therapy is effective in some cases, synergy studies should be encouraged.

In conclusion, *C. lamprauges* is a new addition to the species of *Conidiobolus* recognized as capable of causing vascular invasion and fatal disseminated disease in humans.

Nucleotide sequence accession numbers. Sequence data have been deposited in the GenBank database under accession numbers HM593511 (ITS sequence) and HM593512 (D1/D2 sequence).

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